PHARMACOKINETICS OF FLAXSEED LIGNANS IN THE RAT

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by

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ABSTRACT

Secoisolariciresinol diglucoside (SDG) is the principal lignan of the flaxseed. In the human body it is metabolized to secoisolariciresinol (SECO) and then to enterodiol (ED) and enterolactone (EL). It has been shown that these compounds help to prevent the development of some hormone dependent diseases (breast, prostate cancers) and type II diabetes. Given numerous health benefits, evaluation of lignan pharmacokinetics is critical to understanding their pharmacology. This research aimed to assess SECO pharmacokinetic parameters in the rat.

The first objective was to isolate pure SDG and SECO sufficient for a pharmacokinetic study. SDG (≥95% purity) was obtained from SDG (40% purity) by preparative HPLC. SECO (≥95% purity) was produced from SDG by acid hydrolysis.

The second objective was to develop and validate an HPLC method with fluorescence detection suitable for pharmacokinetic applications. The method is specific for SECO, ED, and EL quantification in rat serum. Separation is achieved with a C_{18} reversed-phase column under gradient mobile phase conditions, consisting of water and acetonitrile buffered with 0.1% formic acid. Analytes are extracted with diethyl ether and 7-hydroxycoumarin serves as an internal standard. Calibration curves are linear from 0.01 to 10 μg/mL for SECO/ED and 0.05-10 μg/mL for EL. Accuracy and precision are within FDA specified limits.

The third objective was to assess the pharmacokinetics of SECO after a single intravenous (20mg/kg) and oral bolus (40 mg/kg) administration in rat. SECO pharmacokinetic parameters were assessed based on a 12-hour study in Wistar male rats (n=12). The results were reported as mean ± SD: systemic clearance 3.1±1.0 L/h, volume of distribution 17.7±8.3 L, half-life 4.7±3.6 h, and oral bioavailability 26 %. SECO undergoes enterohepatic recirculation and exhibits two-compartment model characteristics with large volume of distribution and low oral bioavailability. An additional 48-hour study indicated that SECO is present in systemic

circulation at least for 21 hours after dosing. ED was detected and quantified in the 8-24 hours samples, while EL was detected in 15-36 hours samples after SECO administration.

Additional pharmacokinetic studies with ED and EL are necessary to understand which lignan form may mediate the beneficial health effects.

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LIST OF ABBREVIATIONS

7-HC	7-Hydroxycoumarin
ACN	Acetonitrile
ASECO	Anhydrosecoisolariciresinol
AUC	Area Under the Curve
AUMC	Area Under the First Moment Curve
C	Concentration
Cl_s	Systemic Clearance
CouAG	p-Coumaric Acid Glucoside
DMBA	Dimethylbenzanthracene
dt	Change in time
ED	Enterodiol
EGFR	Epidermal Growth Factor Receptor
EL	Enterolactone
ER	Estrogen Receptor
F_{oral}	Oral Bioavailability
FeAG	Ferulic Acid Glucoside
GC	Gas Chromatography
HDG	Herbacetin Diglucoside
HDL-C	High-Density Lipoprotein Cholesterol
HMGA	3-Hydroxy-3-Methyl-Glutaric Acid
HPLC	High Performance Liquid Chromatography
HCl	Hydrochloric Acid
HQC	High Quality Control
IGF-I	Insulin-Like Growth Factor-I
IS	Internal Standard
IV	Intravenous
k	Elimination Rate Constant
LDL-C	Low-Density Lipoprotein Cholesterol
LLOQ	Lower Limit of Quantification
LOD	Limit of Detection
LQC	Low Quality Control

MRT	Mean Residence Time
MQC	Middle Quality Control
NaOH	Sodium Hydroxide
PgR	Progesterone Receptor
PK	Pharmacokinetics
QC	Quality Control Sample
SDG	Secoisolariciresinol Diglucoside
SECO	Secoisolariciresinol
SMG	Secoisolariciresinol Monoglucoside
t	Time
$t_{1/2}$	Half-life
TC	Total Cholesterol
TEB	Terminal End Bud
V_d	Volume of Distribution
V_{ss}	Volume of Distribution at Steady State
VEGF	Vascular Endothelial Growth Factor
UPLC	Ultra Performance Liquid Chromatography

1. INTRODUCTION

Flaxseed is a major source of dietary intake of the lignan secoisolariciresinol diglucoside (SDG). Following oral consumption, SDG is converted to several metabolites: secoisolariciresinol (SECO), enterodiol (ED) and enterolactone (EL) (Section 2.2). Numerous animal and human studies indicate that flax lignans may be protective against breast, colorectal and prostate cancers (Section 2.3). It was also shown that consumption of SDG-rich flax extract could decrease elevated cholesterol and glucose in plasma, key components of cardiovascular health. Most of these studies are focused on EL and ED as bioactive molecules, although experimental design often involves consumption of ground flaxseed, flax flour, or SDG. Consequently, subjects are exposed to SECO as well (Section 2.3). Recent studies suggest that SECO is found in detectable quantities in plasma of many species, making it another potentially bioactive molecule. Moreover, Dr. Alcorn's laboratory has established a hypercholesterolemic rat model and showed that chronic daily oral administration of SDG or SECO reduced total cholesterol within normal ranges in rats fed a high cholesterol diet[1]. Therefore, knowledge of SECO pharmacokinetics will help understand the pharmacology of lignans.

Little information is available on SECO bioavailability and disposition (Section 2.4). The aim of my project is to enhance our understanding of SECO pharmacokinetics in the rat model. For this purpose, SECO pharmacokinetic parameters will be assessed after single intravenous and oral bolus administration in rats (Section 3.3). The pharmacokinetic study will also require isolation of pure SECO (Section 3.1) and development of a sensitive and simple method for lignan quantification in rat serum (Section 3.2).

2. LITERATURE REVIEW

2.1 Flaxseed, General Description and Composition

Common flax or *Linum usitatissimum* is one of the oldest cultivated plants, with cultivation starting approximately 8,000 years ago in the region of Tigris and Euphrates rivers[2]. From ancient times it was used as a source of oil and fiber for weaving clothes. Today, long-stem varieties of flax are cultivated for linen production, while short-stem flax is cultivated for flaxseed oil, which is widely used in production of linoleum, stains, and paints[3]. In Canada, flax is one of the major crops, and Canada is the leader in oilseed flax production and export since 1994[4].

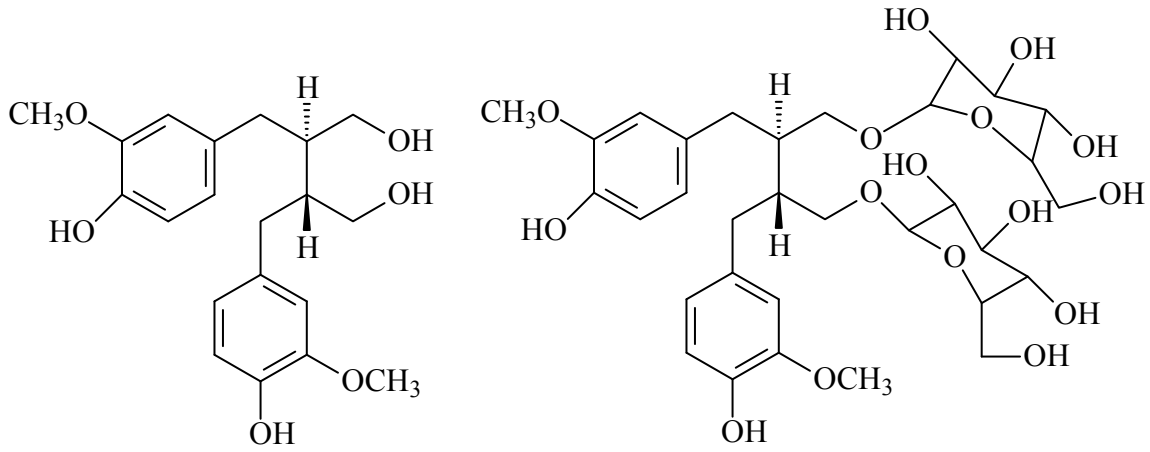
Flaxseed (oilseed variety) has about 40% lipids, 30% dietary fiber, 20% proteins, 4% ash, and 6% moisture (on whole seed basis)[2,3]. The lipid fraction of flaxseed consists of α-linolenic acid (ALA), linoleic, ricinoleic, and palmitic acids. ALA makes up to 55% of the total flax oil. Flaxseed has high content of polysaccharidic mucilage (6 to 8% by dry weight), which consists of acidic and neutral polysaccharides. A number of phytochemicals, such as lignans, cyanogenic glycosides, phenolic acids, isoprenoids, and flavonoids, are also present in flaxseed[2]. In addition to ALA, the lignans of flaxseed receive significant interest due to their putative health benefits in a number of chronic disease states.

2.2 Lignans

Lignans are a class of phytochemicals found in many plant species. Structurally lignans are dimeric compounds that consist of two phenlylpropanoid C_6-C_3 units coupled at the β and β' carbon atoms[5]. In the plant the biological role of the lignans is still unclear, but it is assumed that they control the growth of the plant and provide protection from diseases and pests[5,6]. Lignans are found in all plant parts, including roots, flowers, fruit, seeds, and stems[5,6]. Biosynthesis of lignans is not yet understood, but they are presumed to be related to lignins[5]. According to recent findings, lignans are formed via dimerization of monolignols, allylphenols, or hydrocinnamic acids, depending upon the skeletal subclass formed[3,5,7].

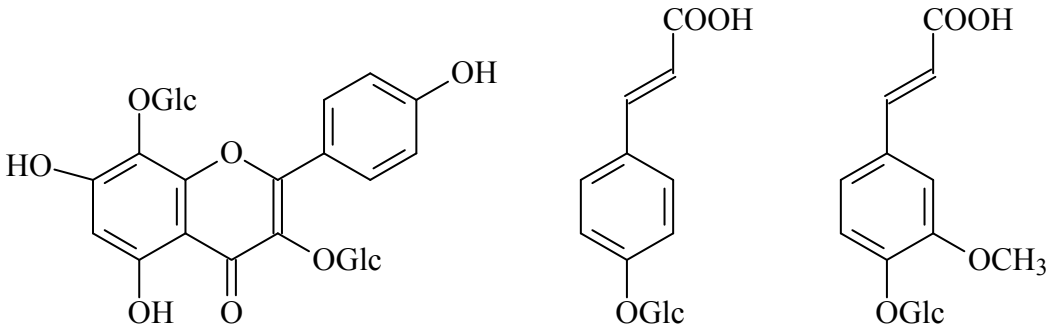
2.2.1 Flaxseed Lignans

Flaxseed contains a number of lignans such as secoisolariciresinol diglucoside (SDG), secoisolariciresinol (SECO), isolariciresinol, pinoresinol, and matairesinol, which are concentrated in the outer layer of the seed and exist in the form of glucosides[3,8]. The main lignan of the flaxseed is SDG (9-30 mg/g of defatted flax meal), and flax is the richest source of SDG among foods (Figure 2.2.1)[2]. SDG is present in flax as a part of a soluble ester-linked complex that contains 3-hydroxy-3-methyl-glutaric acid (HMGA) and a number of cinnamic acid glucosides[2,9]. Recent studies by Struijs *et al.* showed that the lignan macromolecule consists of 62% SDG, 5.7% herbacetin diglucoside (HDG), 12.2% p-coumaric acid glucoside (CouAG), 11% HMGA, and 9% ferulic acid glucoside (FeAG) (w/w) (Figure 2.2.2). They also suggest that average lignan macromolecule has three backbone units (SDG or HDG) with a linker (HMGA) and terminal units (CouAG or FeAG)[10,11]. The chain length may vary from one to seven SDG moieties (Figure 2.2.3).

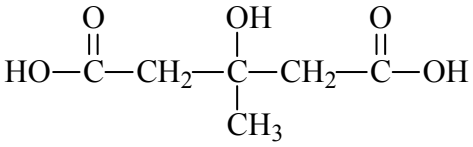


SECO SDG

Figure 2.2.1 Chemical structures of secoisolariciresinol (SECO) and secoisolariciresinol diglucoside (SDG).



HDG CouAG FeAG



HMGA

Figure 2.2.2 Chemical structures of the constituents of the flaxseed lignan macromolecule: HDG, CouAG, FeAG, and HMGA.

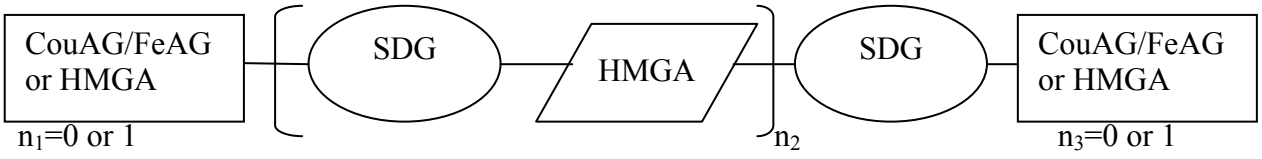
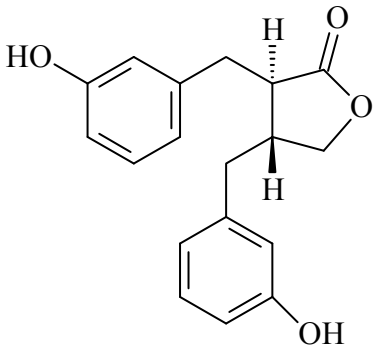


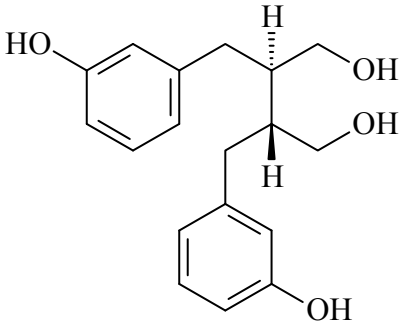
Figure 2.2.3 Schematic representation of the flaxseed lignan macromolecule suggested by Struijs *et al*[11]. The backbone units are SDG or HDG (1 out of 11units) coupled with linker HMGA through the ester bonds and CouAG , FeAG or HMGA as terminal units ($n_1+n_2+n_3 \geq 1$).

2.2.2 Mammalian lignans

Mammalian lignans, or enterolignans, were identified independently by two groups of researchers during gas chromatographic –mass spectrometric analysis of urinary steroid extracts from human and animal species as glucuronic and sulfuric acids conjugates[12-15]. Enterolignans, enterodiol (ED) and enterolactone (EL), have also been found in human plasma, saliva, faeces, semen and prostatic fluids[5]. Axelson *et al.* established that enterolignans are formed by intestinal microflora from dietary sources rich in plant lignans, among which flaxseed had the highest lignan content[16,17]. From flaxseed, the main precursors of ED and EL are SDG, SECO, and matairesinol[3]. *In vitro* incubation with human fecal microflora showed that other plant lignans, lariciresinol, pinoresinol, syringaresinol, and 7-hydroxymatairesinol, could also be converted to ED and EL[18]. Figure 2.2.4 represents the chemical structures of ED and EL which, unlike lignans of plant origin, carry a hydroxyl group in the *meta* position rather than *para* position of aromatic ring[13]. Their identification following flaxseed consumption suggests a role for the enterolignans in the possible health benefits associated with flaxseed lignans.



Enterolactone
2,3-bis-(3'-hydroxybenzyl)-butyrolactone



Enterodiol
2,3-bis-(3'-hydroxybenzyl)-butane-1,4-diol

Figure 2.2.4 Chemical structures of enterolactone (EL) and enterodiol (ED).

2.3 Health Benefits and Biological Activity

Biological activity and potential health benefits of flaxseed lignans have been assessed in numerous *in vitro* and *in vivo* studies. Flaxseed lignans and their metabolites may be protective against some cancers (breast, endometrial, prostate, colorectal), and may reduce risk for cardiovascular disease, hyperlipidemia and type II diabetes[19-22].

2.3.1 Breast Cancer

A series of studies on flaxseed, lignans, and breast cancer were conducted in Sprague Dawley female rats[23-26]. Animals were supplemented with flaxseed flour, defatted flaxseed meal, or SDG at 2.5%, 5%, or 10% levels for up to 22 weeks and different stages of mammary tumorigenesis were assessed. In the flaxseed group, 24 h after injection with carcinogen dimethylbenzanthracene (DMBA) cell proliferation was reduced by 38.8-55.4% and nuclear abberations were reduced by 58.8-65.9% in the female rat mammary gland[23]. Rats with established DMBA-induced tumors supplemented with flaxseed or SDG for 7 weeks had 50% reduction in tumor volume compared to control. A significant negative correlation was observed between excretion of lignans (ED and EL) in urine and tumor volume[24]. Similarly, flaxseed or SDG supplementation decreased invasiveness and grade of N-methyl-N-nitrosourea-induced mammary tumors in rats[25]. Additionally, reduced plasma levels of insulin-like growth factor-I (IGF-I), which is associated with increased breast cancer risk, were observed in rats supplemented with flaxseed or SDG[26]. Reduction of terminal end bud (TEB) structures (most susceptible to carcinogens) in rat mammary gland was observed after lifetime, gestation or lactation exposure to flaxseed or SDG, suggesting their protective role against mammary cancer[27,28]. Moreover, rats exposed to flaxseed or SDG during lactation had significantly lower tumor incidence, tumor load, and mean tumor size and tumor number induced by DMBA when compared to control[29]. An additional study showed that reduction of TEB structures occurs through epidermal growth factor receptor (EGFR) and estrogen receptor (ER) signaling[30].

Chen *et al.*[31] assessed the role of flaxseed and SDG diets in mice with established estrogen-negative human breast cancer tumors (MDA-MB-35). Mice supplemented with 10% flaxseed for 15 weeks had significant reduction in tumor growth rate and 45% reduction in total incidence of metastasis compared to control group. Expression of IGF-I and epidermal growth factor receptor (EGFR) in the primary tumor were also significantly lower in the flaxseed group[31]. Moreover, a separate study with similar setup indicated that extracellular levels of vascular endothelial

growth factor (VEGF), key factor in promotion of tumor angiogenesis, were also significantly reduced[32]. Flaxseed (10%) and SDG (equivalent to 10% flaxseed) supplementation for 7 weeks reduced incidence of metastases in mice after excision of primary MDA-MB-35 tumor[33].

The effect of flaxseed supplementation was also studied in ovariectomized mice with established estrogen receptor-positive human breast cancer (MCF-7) tumors at low and high levels of circulating estrogens. Tumor size regressed by 74% after 6 weeks on 10% flaxseed diet at low estrogen levels. At high estrogen levels tumor growth was inhibited by 22% in the flaxseed group, and by 50% while in combination with tamoxifen (tamoxifen alone inhibited tumor growth only by 41%)[34]. An additional experiment confirmed that flaxseed diet does not stimulate tumor growth at low estrogen levels, while tamoxifen and soy protein do[34-36]. Moreover, analysis of tumor biomarkers in ovariectomized mice with established MCF-7 tumors at high levels of estrogen indicated that inhibition of tumor growth resulted from decreased cell proliferation and increased cell apoptosis in the flaxseed groups in a dose-dependent manner[37]. Expression of progesterone receptor (PgR) and IGF-I were also significantly reduced compared to control[37]. A recent study compared effects of flaxseed diet and equivalent supplementation of pure SDG in ovariectomized mice with established MCF-7 tumors with low estrogen levels[38]. Both treatments significantly inhibited cell proliferation and induced apoptosis, as well as significantly decreased mRNA expression of estrogen-responsive genes cyclin D1, pS2, ERα, ERβ, and biomarkers in growth factor-signaling pathways such as EGFR and IGF-I receptor, suggesting that SDG has similar effect as flaxseed in reducing tumor growth[38]. Saarinen et al. reported that SDG is also accessible to tumor tissues after oral administration of tritium labeled SDG to mice with established MCF-7 tumors[39].

The protective role of ED and EL against breast cancer was assessed *in vitro* in two estrogen receptor negative cell lines (MDA-MB-231 and MDA-MB-435). ED and EL inhibited steps involved in the metastatic cascade such as cell migration, invasion, and adhesion to Matrigel or extracellular matrices in a dose-dependent manner[40]. In MCF-7 estrogen receptor positive cell line ED and EL significantly inhibited aromatase and 17β-hydroxysteroid dehydrogenase (17β-HSD), with ED being more potent aromatase inhibitor, while EL being 17β-HSD inhibitor[41]. Subcutaneous injections of ED or EL (10mg/kg) for 22 weeks in ovariectomized mice with established MCF-7 tumors with low estrogen levels did not promote tumor growth compared to negative control and significantly induced tumor cell apoptosis[42]. Ovariectomized mice with established MCF-7 tumors with high estrogen levels injected

subcutaneously with ED or EL (15 mg/kg) for 5 weeks showed decreased tumor growth and decreased extracellular levels of VEGF[43].

A number of case-control studies in humans examined association between serum EL and risk of breast cancer. An inverse association between serum EL and breast cancer risk was observed among premenopausal and postmenopausal women in Kuopio Breast Cancer Study, Finland[44]. Sonestedt et al. found that high plasma EL is associated with the reduced risk for ERα positive/ERβ negative breast tumors in nested case-control study in Sweden[45]. On the other hand, a prospective study in New York did not support protective role of EL[46].

Population-based studies on phytoestrogen dietary intake (isoflavones and lignans) and breast cancer in Ontario, Canada, indicate that lignan intake is associated with reduced breast cancer risk among overweight premenopausal women[47]. Additionally, adolescent lignan intake may be associated with decreased risk of adult breast cancer[48]. Furthermore, dietary lignan intake has been associated with improved survival among postmenopausal women with breast cancer in the Western New York Exposures and Breast Cancer Study[49].

2.3.2 Melanoma

Two studies investigated the effect of flaxseed and SDG on the lung metastases of the murine melanoma B16BL6 cells in mice. Mice were supplemented with flaxseed or SDG at three concentration levels for two weeks before injection of B16BL6 cells and two weeks after the injection. The number of tumors in mice fed 2.5%, 5% and 10% flaxseed was 32%, 54% and 63% lower than in control group[50]. Dietary supplementation of SDG also reduced the number of tumors in treated groups compared with the control, as well as decreased tumor cross-sectional area and tumor volume in a dose-dependent manner[51].

2.3.3 Endometrial Cancer and Uterine Fibroids

A case-control study in the USA showed a negative association between dietary intake of lignans and endometrial cancer, which was statistically significant in postmenopausal women[52]. On the other hand, in the case-control study nested within three prospective studies in Sweden, Italy, and USA no correlation was found between serum EL levels and endometrial cancer[53]. Additionally, in a case-control study in the United States a modest inverse association was observed between lignan excretion and risk of uterine fibroids (hormonally responsive benign tumors)[54].

2.3.4 Prostate Cancer and Benign Prostatic Hyperplasia

Lin et al. investigated the effect of ED and EL on prostatic carcinoma *in vitro*[55]. Both lignans significantly inhibited the growth of three human prostate cancer cell lines (PC-3, DU-145 and LNCaP), with EL being more potent inhibitor (IC_{50} = 57 μM for LNCaP cells) compared to ED (IC_{50} = 100 μM for LNCaP cells)[55]. The same research group studied the effect of 5% flaxseed diet on prostatic neoplasia in the transgenic adenocarcinoma mouse prostate model[56]. Mice supplemented with flaxseed for 30 weeks had significantly less aggressive tumors compared to control, reduced number of lung and lymph node metastases, significantly reduced cell proliferation and increased cell apoptosis[56]. Similar results were observed in rats with Dunning R3327 PAP prostate tumors supplemented with rye bran rich in lignans[57]. The number of palpable tumors and tumor volume were significantly lower in the rye bran group compared to control after 14 and 16 weeks of supplementation[57].

Human data on the role of lignans in prostate cancer are controversial. Stattin et al. found no association between serum EL and prostate cancer incidence in a nested case-control study conducted in Finland, Norway and Sweden[58]. However, men with very low plasma levels of EL had a significantly higher risk of prostate cancer[59]. In a case-control study on diet and prostate cancer in western New York a reduced risk of prostate cancer was observed in men consuming diet rich in lignans[60]. Another dietary study in Sweden found that intermediate levels of plasma EL were inversely associated with prostate cancer risk[61]. Furthermore, recent analysis of individual patient data from 12 prospective studies by Roddam et al. found an association between high circulating plasma IGF-I and prostate cancer risk[62]. SDG was shown to reduce IGF-I levels in rats and could be protective against prostate cancer[26].

Positive effect of lignans on benign prostatic hyperplasia was observed in a randomized double-blind placebo-controlled study in 87 subjects treated with SDG (300 or 600 mg/day) for 4 months. After completion of the study the International Prostate Symptom Score decreased significantly, while Quality of Life improved compared to control group. Therapeutic efficacy of SDG was comparable with 5α-reductase inhibitors and α1A-adrenoreceptor blockers, commonly used for the treatment of benign prostatic hyperplasia[63].

2.3.5 Colorectal Cancer

The protective role of flaxseed diet against colon cancer was observed in rats fed with flaxseed flour or flaxseed meal (5% or 10%) for 4 weeks following a single injection of

carcinogen azoxymethane. By the end of the experiment the total number of aberrant crypts and foci (early markers of cancer risk) was significantly reduced by 41-53% and 48-57%, respectively[64]. Additional study on long-term (100 days) exposure to flaxseed, defatted flaxseed or SDG in rats injected with azoxymethane had similar results, indicating that protective effect of flaxseed could be attributed to SDG[65].

EL and ED had an inhibitory effect on four colon cancer cell lines (LS174T, Caco-2, HCT-15, T-84) *in vitro*, with EL being more effective than ED[66]. Moreover, EL suppressed growth of Colo 201 human colon cancer cells *in vitro* and *in vivo* (10 mg/kg EL injected subcutaneously to athymic mice with transplanted Colo201tumors for 18 days), by inducing apoptosis and decreasing cell proliferation[67]. Another study in multiple intestinal neoplasia mice, a model for colon tumorigenesis, did not show protective effect of 0.5% defatted flaxseed meal diet, although there was a tendency for decreased number of colon adenomas in the flaxseed group[68].

In a Dutch case-control study in humans, plasma levels of ED and EL were inversely associated with colorectal adenoma (colorectal cancer precursor) risk[69]. Another case-control study in Ontario, Canada, found that dietary lignan intake is significantly associated with reduced colorectal cancer risk[70].

2.3.6 Cardiovascular Health and Diabetes

Cardioprotective role of flaxseed and lignans was assessed in a series of studies in hypercholesterolemic rabbits. Animals on atherogenic diet were supplemented with type II flaxseed low in α-linolenic acid for 8 weeks[71]. Type II flaxseed was chosen to evaluate effect of the lignan component of flax on development of atherosclerosis. The development of aortic atherosclerosis was reduced by 69% in the flaxseed group. Serum total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) were lower compared to control, while high-density lipoprotein cholesterol (HDL-C) was not affected[71]. Additional study with flaxseed oil suggested that the oil component of flax has no effect on serum lipids or extent of atherosclerosis in rabbits[72]. Furthermore, hypercholesterolemic rabbits supplemented for 2 months with SDG macromolecule complex (40 mg/kg per day) had decreased serum TC, LDL-C, and TC/HDL-C levels, while no changes were observed in normocholesterolemic rabbits[73]. Development of aortic atherosclerosis was reduced by 35%[73]. Protection against atherosclerosis was also observed in a study involving rabbits supplemented with an atherogenic diet for 4 months with SDG complex supplementation during the last 2 months of the experiment[74].

Felmlee *et al.*[1] observed a dose-dependent reduction in the rate of body-weight gain, serum TC and LDL-C, and hepatic lipid accumulation in hypercholesterolemic rats supplemented with equimolar quantities of SDG (3 or 6 mg/kg) and SECO (1.6 or 3.2 mg/kg) for 4 weeks[1]. Another study in rats fed high cholesterol diet for 8 weeks followed by oral administration of SDG (20 mg/kg) for 2 weeks showed a significant reduction in TC, LDL-C, triglycerides and increase in HDL-C levels, compared to control[75]. Protective effect of SDG against ischemia-reperfusion injury *ex vivo* was also observed in the same study, as well as significant improvement in left ventrical functions in induced myocardial infarction *in vivo*[75].

Prasad *et al.*[76,77] assessed the protective role of SDG against type II diabetes in rats. Incidence of streptozotocin-induced diabetes in rats supplemented with 22 mg/kg SDG for 21 days was reduced by 75%[76]. In Zucker diabetic fatty rats (model for type II diabetes) treated with SDG (40 mg/kg orally) starting at age 6 weeks, development of diabetes was delayed in 80% of animals in the treatment group[77].

Recent studies showed beneficial effect of SDG on hypercholesterolemia and hyperglycemia in humans. In a randomized double-blind placebo-controlled study 55 hypercholesterolemic subjects were given SDG (300 and 600 mg/day) for 8 weeks. By the end of experiment SDG significantly reduced plasma TC, LDL-C, and fasting glucose levels in a dose-dependent manner[78]. Another randomized double-blind placebo-controlled study in 72 diabetic patients with mild hypercholesterolemia given 360 mg/ day of SDG for 12 weeks suggested that SDG modulates levels of C-reactive protein, a marker for chronic inflammation and one of the key factors in pathogenesis of type II diabetes[79].

Vanharanta *et al.* reported that high serum EL levels were associated with reduced coronary heart disease-related and cardiovascular disease-related mortality in middle-aged Finnish men in Kuopio Ischaemic Heart Disease risk factor study[80]. On the other hand, Kuijsten *et al.* did not associate high plasma ED and EL with a reduced risk on nonfatal myocardial infarction in prospective case-control study in Netherlands[81]. Peterson *et al*. in their recent review of the epidemiological studies on lignan dietary intake suggested that lignans might be protective against cardiovascular disease, although more research is needed[22].

2.4 Pharmacokinetics of Flaxseed Lignans

An understanding of lignan pharmacokinetics (PK) is critical for assessments of biological activity, mechanism of action and potential therapeutic application (i.e. size and frequency of dose, therapeutic concentration). To date, limited PK data exists for the flaxseed lignans. For a long time only EL was in the scope of interest of researchers. Information on lignan bioavailability and serum and tissue levels of the various lignan forms and their oxidative or phase II metabolites is limited. Available evidence suggests lignans undergo extensive enterohepatic recirculation such that the liver and gastrointestinal tract are exposed to high levels of the lignans.

2.4.1 Absorption and Gastrointestinal Metabolism

Shortly after discovery of mammalian lignans studies in rats and humans confirmed that ED and EL were produced by intestinal microflora and were metabolites of SDG from flaxseed (mammalian lignans were not detected in germ-free rats)[16,17]. A simplified scheme for the conversion of SDG into ED and EL was first proposed by Axelson *et al.* in 1982[17], and was clarified in recent studies (Figure 2.4.1)[82-88].

To date, research indicates that following flaxseed consumption the lignan macromolecule breaks down to SDG and various combinations of SDG and HMGA in the acidic environment of stomach and SDG is completely released from macromolecule in the small intestine[85]. Milling and crushing of flaxseed was shown to improve the conversion to enterolignans[89]. The glucose groups on SDG are then hydrolyzed by microbial and intestinal brush border β-glucuronidase or β-glucosidase to produce the aglycone SECO[82,90]. *Bacteroides* and *Clostridium* species are responsible for deglycosylation of SDG in humans[87,91]. Glitso *et al.* suggested the conversion occurs between terminal ileum and caecum in pigs[92]. Similarly, Eeckhaut *et al.* observed SDG conversion into SECO in the ascending colon compartment in the human artificial digestion model, which is supported by an earlier study in patients with ileostomies having low plasma lignan levels[85,93]. Then, SECO is partially absorbed into systemic circulation as was shown in a recent study by Laerke *et al.*[94] or is converted to mammalian lignans by gut microflora[86-88]. *In vitro* and *in vivo* studies showed that SECO is first demethylated by *Blautia producta* and *Eubacterium limosum*, and then dehydroxylated by *Eggertella lenta* to produce ED[82,83,86,88,91]. ED is dehydrogenated to EL by recently isolated *Lactonifactor longoviformis*[87,88].

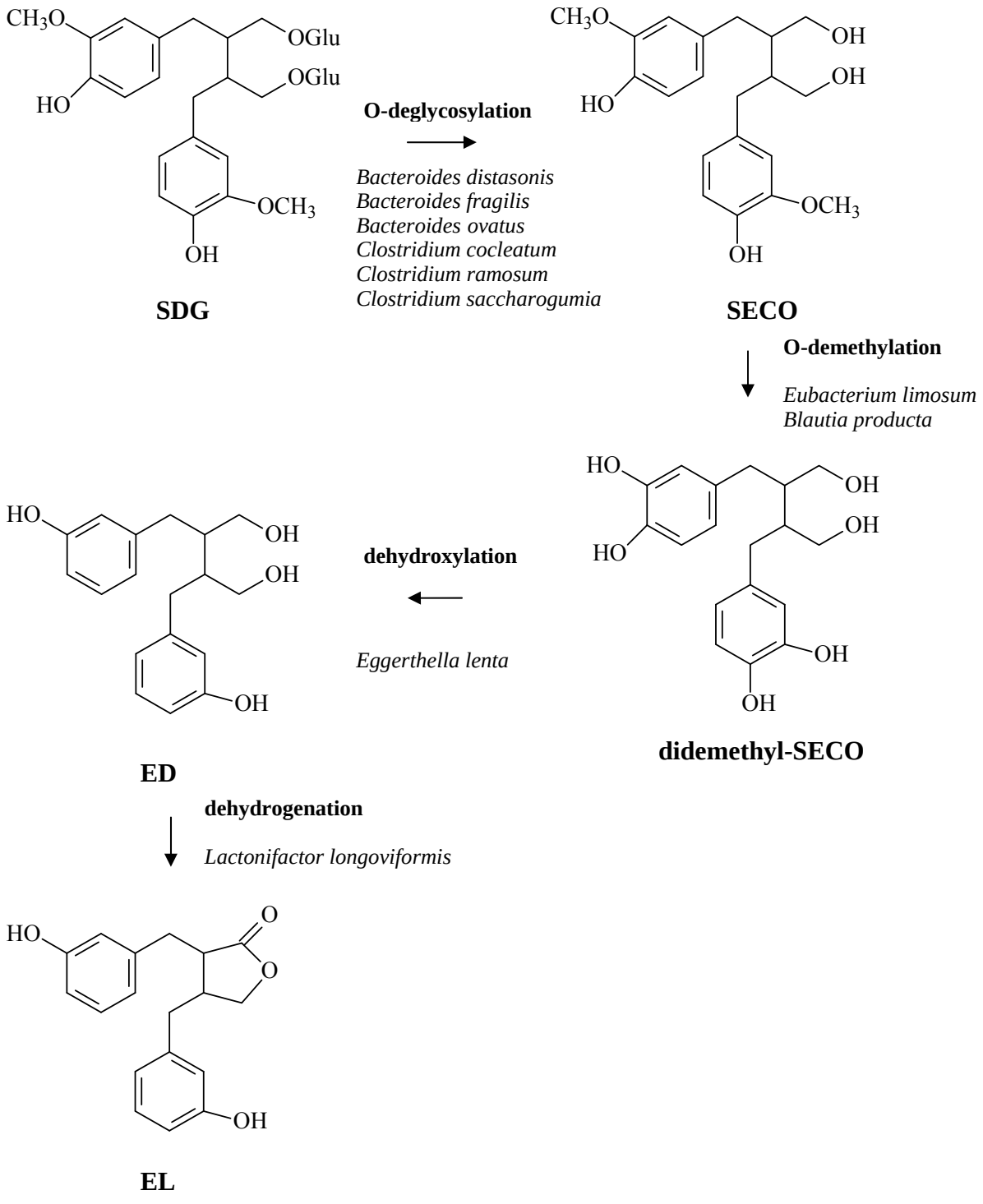


Figure 2.4.1 Proposed metabolic pathway for the conversion of SDG by intestinal microflora (adopted from Wang *et al.*[82]).

Intestinal lignan metabolism varies significantly among the population, which can be stratified into high, moderate and low ED/EL producers[84]. Additionally, *in vitro* SECO incubation with human fecal samples showed that high concentration of *Blautia producta* and bacteria belonging to *Atopobium* group was typical for high EL-producers, while ED production was negatively correlated with abundance of *Clostridium coccoides-Eubacterium rectale* cluster[83,84].

2.4.2 Phase I and Phase II Metabolism

Once absorbed from the intestine, SECO, ED and EL undergo conjugation reactions with glucuronic and sulfuric acids by phase II enzymes. Analysis of urine and plasma in humans suggested that lignans form mainly glucuronides (up to 98%) and smaller quantities of monosulfates and disulfates[15,95,96]. Similar results were obtained *in vitro*, after EL was incubated with human and rhesus monkey hepatocytes with reaction catalyzed by human UGT1A and UGT2B family members[97]. Study in rats also showed that conjugates are excreted into bile and undergo enterohepatic circulation[16]. Another study with human colon epithelial cell lines (CaCo-2 and HT29) suggested that ED and EL could be also conjugated during the uptake in the colon, with EL being more rapidly metabolized than ED[98].

SECO, ED and EL are as well substrates for cytochrome P-450-mediated hydroxylation reactions. *In vitro* studies on lignan oxidative metabolism in human, pig, rhesus monkey, and rat microsomes gave a number of aromatic and aliphatic monohydroxylated derivatives[97,99-101]. Similarly, a number of aromatic and aliphatic hydroxylated metabolites were detected in bile and urine of rats dosed, either orally or intraduodenally, with ED and EL (10 mg/kg)[102]. Interestingly, SECO incubated with human and rat liver microsomes could be metabolized into lariciresinol and isolariciresinol[101]. Nine aromatic metabolites of ED and EL were also identified in urine of humans supplemented with flaxseed for 5 days, although they accounted for less than 5% of the total urinary lignans[103]. A recent study in human and rhesus monkey hepatocytes also indicates that hydroxylation is a minor metabolic pathway for lignans[97].

2.4.3 Distribution

Analysis of lignan levels in serum shows a wide range of concentrations within population. Adlercreutz *et al.*[95] reported higher levels of ED and EL in vegetarian women (ED 1.1-140.3 nmol/L; EL 17.9-1078.2 nmol/L) than in omnivorous women (ED 0-5.6 nmol/L; El

10.4-74.1 nmol/L)[95]. Kilkkinen *et al.*[104] found wide variation in serum EL levels in 2380 Finnish adults (men: 0-95.6 nmol/L; women: 0-182.6 nmol/L)[104]. ED and EL have been also quantified in human saliva, prostatic fluid, and semen[5,105]. Furthermore, study in 7 healthy Japanese women at delivery suggests that lignans freely pass the placental barrier, as maternal plasma ED and EL concentrations significantly correlated with levels in cord plasma and amniotic fluid[106].

Rickard and Thompson assessed tissue lignan distribution in rats over 48 hours after acute and chronic (rats gavaged with 1.5 mg/day of SDG for 10 days) tritium labeled SDG (^{3}H-SDG) treatment[107]. Total lignans were measured as radioactivity by liquid scintillation in16 tissues, as well as blood, urine and faeces. More than 80 % of recovered dose was excreted by 48 hours. Caecum had the highest lignan level throughout the experiment. Other tissues with high lignan concentrations were liver, kidney and uterus. Chronic exposure to SDG resulted in delayed fecal excretion of lignans while increasing levels in liver and adipose tissue, suggesting lignan accumulation in tissues[107]. Similarly, Murray *et al.*[108] observed dose-dependent accumulation of ED and EL in liver, testes, lungs, and prostate in male rats supplemented with linola meal for 7 days[108]. A recent study on lignan tissue distribution in adult male and female rats supported earlier results and also suggested sex-related differences in lignan distribution (female rats had higher lignan concentrations in heart and thymus)[109]. In another study in pigs fed rye bread diet rich in lignans for 58-67 days significant EL concentrations were found in the colon (691.7±93.63 pmol/g), liver (125.0±19.63 pmol/g), and breast tissue (25.8±4.12 pmol/g). EL was also found in brain tissue (2.3±0.41 pmol/g)[94]. In the same study bile analysis showed domination of plant lignans (77%: SECO, matairesinol, and lariciresinol) as opposed to EL (23%), with 6.2±0.9 μmol/L of total lignans[94]. One study in rats gavaged with ^{3}H-SDG also suggested that lignans could be transferred via dam's milk to the offspring, although lignan form was not specified[110].

2.4.4 Excretion

Conjugated lignans are excreted through the urinary and biliary systems[16]. Human dietary studies suggest a dose-dependent increase in ED and EL urinary excretion following increasing flaxseed consumption[111,112]. Following SDG consumption in 12 volunteers 40 % of the ingested dose was excreted within 3 days with the majority as EL[113]. Similar results were obtained in rats gavaged with ^{3}H-SDG where 28-32% of recovered dose was excreted in urine within 48 hours[107]. Total lignan excretion (measured as radioactivity by liquid scintillation) increases

significantly after prolonged exposure to SDG[109]. In another study in rats given SECO (25 mg/kg), ED and EL were the main urinary metabolites with smaller quantities of SECO and traces of lariciresinol and isolariciresinol[114].

Lignan urinary excretion varies significantly within population. Analysis of urine samples of women on habitual diet suggests that urinary EL excretion is the highest (1691 ng/mL), followed by ED (234 ng/mL) and SECO (124 ng/mL), although the range of concentrations is wide (EL: 2-13156 ng/mL; ED: 0-4337 ng/mL; SECO: 0.7-3200 ng/mL)[115].

The early studies by Axelson and Setchell showed that lignans are excreted into human and rat urine mainly as glucuronides (98% for EL and 92% for ED of total lignans), sulfates (2% for EL and 7% for ED of total lignans), and small amounts of disulfates[15,16]. Adlercreutz *et al*.[96] also reported a sulfoglucuronide fraction of ED, EL and matairesinol in human urine[96]. With more advanced methods for lignan analysis small quantities of conjugated SECO, isolariciresinol and lariciresinol were found in urine samples of women on habitual diet[116], as well as syringaresinol and pinoresinol[117].

Little information is available on lignan biliary excretion. In study by Axelson and Setchell rats excreted relatively high amounts of ED (0.4 μg/24h) and EL (14 μg/24h) into bile in the form of glucuronides (up to 99% of total enterolignans)[16]. Traces of mono- and disulfates were also detected. In another study in pigs fed a rye diet, plant lignans accounted for 77% of the total lignans (SECO: 13%), while ED and EL accounted only for 23% of total lignans (6.2±0.9 μmol/L)[94].

After biliary excretion into the duodenum, lignans undergo deconjugation with bacterial β-glucuronidase. Unconjugated lignans could be reabsorbed or be excreted into faeces. As with bile, little is known about fecal excretion of lignans. In rats gavaged with ^{3}H-SDG, 40% to 83% of the administered dose is excreted into faeces[109]. In humans, EL and ED are two major lignans found in faeces[118]. Small quantities of matairesinol were also detected in women on habitual diet[118]. Similar to urinary excretion, flaxseed supplementation caused increased fecal excretion of EL and ED (16- to 32-fold) in women[119]. The slight increase in matairesinol excretion (1.6-fold) was also observed in the same study, suggesting SECO could also be eliminated via faeces. Unfortunately, SECO analysis was not performed at that time[119].

2.4.5 Pharmacokinetic (PK) parameters

Several studies attempted to assess PK parameters of lignans in humans[113,120,121]. Due to

high cost and complexity of obtaining pure lignans in quantities sufficient for conducting the experiment, only one study was done with pure SDG, while the others used sesame seeds and strawberries.

Mazur *et al.*[120] measured EL plasma levels and urinary excretion in 7 volunteers after the consumption of a single dose of strawberries (500 g) equivalent to 11.7 mg of SECO and 0.61 mg of matairesinol[120]. EL appeared in plasma 8 hours after the strawberry meal, reaching maximum at 24 hours. Maximum EL excretion was observed in 25-36 hour urine samples.

Similarly, in 12 volunteers EL and ED appeared in plasma 8-10 hours after the ingestion of a single dose of purified SDG (1.31μmol/kg body weight)[113]. ED maximum plasma concentration was observed 14.8±5.1 hours, while EL maximum was reached 19.7±6.2 hours post dose. Elimination half-life was 4.4±1.3 h for ED and 12.6±5.6 h for EL. The mean residence time for ED was 20.6±5.9 h and 35.8±10.6 h for EL.

Penalvo *et al.*[121] calculated PK parameters for several plant lignans in 4 volunteers after the consumption of a single dose of sesame seeds (50g) equivalent to 186.5 mg of total lignans including 7.4 μg of SECO[121]. Taking into consideration that SECO is a minor constituent of sesame seeds, researchers were able to calculate only absorption half-life (1.45±0.59 h) and time needed to maximum plasma concentration (6.25±2.25 h).The sampling schedule was not appropriate for determination of PK parameters for ED and EL. Similar to other studies, their concentration increased during the period 10-24 h after the ingested dose[121].

2.5 Analytical Methods for Lignan Quantification in Biological Fluids

Analytical methods for lignan determination and quantification reported in the literature usually involve mass spectrometry (MS) or tandem mass spectrometry (MS/MS) coupled with gas or liquid chromatography[3,5,6,122,123]. Procedures vary depending on the nature of analyzed sample. Based on the reviewed studies, quantification of ED and EL in biological fluids, as molecules potentially responsible for observed health benefits, was the main focus of researchers over the years. At the same time, methods for SDG and SECO quantification were developed mainly for analysis of flaxseed and food samples.

2.5.1 Gas Chromatography

The development of lignan analysis in biological fluids started from gas chromatographic-mass spectrometric (GC-MS) urine analysis of steroid compounds[13]. ED and

EL were among first lignans identified and quantified using capillary gas chromatography (GC) with flame ionization detector and GC-MS in urine samples of humans and several animal species[13,15,124], and later in rat bile and plasma[16]. Both forms of lignans, unconjugated and conjugated (glucuronides, mono- and disulfates) were analyzed. Sample preparation involved prior separation of conjugated and unconjugated compounds on ion exchange column followed by conversion to trimethylsilyl (TMS) ether derivatives and GC or GC-MS analysis with 3α-hydroxy-5β-cholestane as internal standard. Lignan conjugates were hydrolyzed enzymatically (β-glucoronidase for glucuronides) or treated with acidified tetrahydrofuran (for sulfates) before reaction with silanization agents. The method was then adapted by Bannwart C. *et al.* for detection and quantification of SECO in human urine[116].

Setchell *et al.* also proposed quantification of ED and EL in urine and serum using stable isotope dilution GC-MS in the selected ion monitoring mode[125]. The method involved hydrolysis with β-glucoronidase from *Helix Pomatia* multiple steps extraction procedure using liquid-solid extraction and ion-exchange chromatography and addition of deuterium-labeled standards ($[D_2]$-EL and $[D_2]$-ED). A similar method was reported by Adlercreutz *et al*. for quantification of ED, EL[118], as well as isoflavonoids in human urine, where $[D_6]$-EL and $[D_6]$-ED were used as standards. The method was then modified for analysis of plasma[95] and faeces[126] in man, and recently SECO[127] was included into analysis.

Grace *et al.*[128] proposed an assay for ED and EL in low volumes of urine (200μL) by GC-MS in selected ion monitoring mode using $^{13}C_3$-labeled standards. Sample preparation requires reaction of deconjugation with β-glucoronidase/sulfatase, followed by one-step solid-phase extraction (SPE), and silanization of unconjugated ED and EL[128]. Sensitivity of the method is reported to be 1.2 ng/mL for ED and 5.3 ng/mL for EL.

2.5.2 Liquid Chromatography

Taking into consideration that GC methods for lignan analysis require laborious and time consuming clean-up procedure, and derivatization of analytes to make them volatile, a number of methods using high performance liquid chromatography (HPLC) were also developed.

An HPLC method with coulometric electrode array detection was reported for monitoring of phytoestrogens in plasma and urine, including SECO, ED, and EL[129,117]. Sodium acetate buffer-methanol-acetonitrile mixture was used as an eluent. Sample preparation included glucuronidase pretreatment and double extraction with diethyl ether. Run time was 85 min. The

method was later optimized for quantification of 11 lignans in human plasma, with run time reduced to 50 min and limit of detection (LOD) SECO 1.36 nmol/L, ED 0.85 nmol/L, and EL 1.55 nmol/L, but plasma sample needed additional clean-up using ion-exchange chromatography[130].

Several sensitive and selective methods using HPLC coupled with tandem mass spectrometry were published recently. An HPLC-MS/MS method with heated nebulizer atmospheric pressure chemical ionization (HN-APCI) was developed for the determination of phytoestrogens, including ED and EL, in human serum and urine. Sample preparation includes pretreatment with β-glucuronidase/sulfatase, SPE, and addition of deuterium-labeled analytes as internal standards (LOD 0.1-1.1 ng/mL for serum and 0.5-3.3 ng/mL for urine)[131]. Another highly sensitive (LOD 10 pg/mL) HPLC-MS/MS method using triple quadrupole mass spectrometer in negative ion electrospray mode was reported for quantification of isoflavones, ED and EL, and later SECO, in human serum and urine using triply C^{13}-labeled standards[132,115]. Sample preparation also requires prior deconjugation with β-glucuronidase and SPE. The method was later optimized by Kuijsten *et al.* for the measurement of ED and EL only in human plasma, by reducing time needed for reaction of deconjugation from 12 to 4 hours[133].

2.5.3 Time-Resolved Fluoroimmunoassay

A time-resolved fluoroimmunoassay (TRF) was developed for screening of the large number of samples, necessary in epidemiological studies. An assay was developed only for EL quantification in plasma and urine[134-136]. Briefly, conjugated EL is enzymatically hydrolyzed by β-glucoronidase/sulfatase, EL is extracted and then incubated with specific rabbit antibody and europium-labeled EL derivative, where analyte and labeled compound are competing for binding with antibody. After the bioaffinity reaction is completed, europium is dissociated from the antibody-EL derivative complex and its fluorescence is measured at a fixed time after the excitation of fluorophore. The method is sensitive (1.5 nmol/L), specific, though there is some cross-reactivity with ED, and allows the processing of high number of samples[134].

2.6 Methods of Lignan Analysis in Flax and Food samples

An increasing interest in mammalian lignans, ED and EL, and their biological role led to the development of methods for quantitative analysis of SDG and SECO in food, suitable to conduct dietary intake studies (since SDG and SECO were found to be the main precursors of ED and EL). Most of the reported methods for lignan quantification in flax and food samples use different types of hydrolysis reactions to convert lignans from glycoside to aglycone form with subsequent analysis of aglycones using GS-MS or HPLC-MS.

In 1991 Thompson *et al.*[137] adapted capillary GC-MS method reported by Fotsis *et al.*[124] to measure ED and EL that could be produced from various foods[137]. Food samples were fermented *in vitro* by human colonic microflora prior to analysis.

In 1995 Obermeyer *et al.*[138] quantified SECO in flax and flax meal using HPLC with UV detection at 280 nm and coumarin as internal standard. Samples were hydrolyzed with β-glucuronidase, rinsed with acetonitrile, and subjected to SPE before HPLC analysis[138].

In 1996 Mazur *et al.*[139] proposed acid hydrolysis for analysis of SECO and matairesinol in flax and selected foods along with simultaneous quantification of several isoflavonoids by isotope dilution GC-MS[139]. Sample preparation involved enzymatic hydrolysis and ether extraction of isoflavonoids followed by acid hydrolysis with hydrochloric acid (HCl) for 2.5 hours and ether extraction of lignan aglycones. Then ether fractions were purified by ion-exchange chromatography, silylated and analyzed using GC-MS. Deuterated analytes were used as internal standards. Sensitivity of the method was reported to be 2-3 μg/100g of the sample. Later Liggins *et al.*[140] modified the method for quantification of SECO, shonanin and matairesinol. Sample preparation included acid hydrolysis with HCl for up to 3 hours, triple extraction of lignan aglycones with ethyl acetate and methyl *ter*t-butyl ether mixture, followed by silanization and GC-MS analysis. Anthraflavic acid was used as internal standard[140]. Researchers reported more than 4-times higher levels of SECO in flaxseed, than those obtained by Obermeyer *et al*.[138], since β-glucuronidase did not completely hydrolyze lignan glycosides as opposed to hydrochloric acid. The drawbacks of the method included formation of the artifacts during acid hydrolysis, when SECO is dehydrated to anhydrosecoisolariciresinol (ASECO) which is structurally similar to the naturally occurring lignan shonanin. Therefore, lignan levels could only be reported as the total concentration of SECO and shonanin. Also optimal time for acid hydrolysis varied depending on a food matrix[140].

Considering instability of lignans during acid hydrolysis, Milder *et al.*[141] combined

enzyme and alkaline hydrolysis to quantify SECO, lariciresinol, matairesinol, and pinoresinol in foods using liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) with atmospheric pressure chemical ionization (APCI). Samples were treated with 0.3M sodium hydroxide (NaOH) at 60°C for 1 h, followed by overnight hydrolysis with β-glucuronidase, and extracted twice with diethyl ether before analysis. Deuterated SECO and matairesinol were used as standards. Sensitivity of the method was reported to be 4-10 μg/100 g of sample[141]. Alkaline hydrolysis of the sample improved lignan yield by 80% compared to enzymatic hydrolysis alone.

Later Penalvo *et al.*[142] adapted sample pretreatment procedure used by Milder *et al.*[141] for quantification of SECO, matairesinol, lariciresinol, pinoresinol, medioresinol, and syringaresinol by isotope dilution GC-MS in food items. $^{13}C_3$-labeled lignans were used as internal standards and sensitivity of the method was reported to be 6-12 μg/100 g of sample.

Several methods for SDG quantification have been reported over the years. Muir and Westcott[143] suggested HPLC method with UV detection at 280 nm after base hydrolysis of flax and flax containing food items. Samples were defatted by hexane and subjected to alkaline hydrolysis with 0.5 N NaOH for 3 hours before HPLC analysis. SDG was separated using 1% acetic acid and methanol as mobile phase, with gradient applied, and 60 min runtime. Similarly, Johnsson *et al.*[144] reported HPLC method with UV detection at 280 nm for SDG analysis in defatted flaxseed flour. Sample was extracted with 1,4-dioxane-methanol mixture, hydrolyzed with 0.3 M NaOH for 48 hours, and purified by SPE. Runtime was reduced to 32 min with 5% acetonitrile in 0.01M phosphate buffer and acetonitrile as mobile phase.

2.7 Purpose and Objectives

2.7.1 Purpose

Lignans are reported to have numerous health benefits. For many years enterolignans, and especially EL, were in the scope of researchers' interest as possessing those benefits. Consequently, analytical methods for lignan quantification in biological fluids were developed firstly for enterolignans. SDG and SECO were viewed as precursors of ED and EL, and were analyzed mostly in various foods to assess dietary lignan intake. Recent studies suggest that SECO is found in detectable quantities in plasma of many species. Furthermore, many studies on biological activity of lignans were conducted using flaxseed, due to the expensive and labourious procedure of obtaining pure substances in quantities sufficient for experiment. That means, study participants were exposed to SDG, SECO, ED, and EL, leaving unanswered the question of

which molecule is biologically active. Consequently, knowledge of SECO pharmacokinetics is critical for understanding the pharmacology of lignans.

Few studies have assessed the pharmacokinetics of lignans and those conducted provide an incomplete overview of lignan bioavailability and disposition. My laboratory has established a hypercholesterolemic rat model and showed that chronic daily oral administration of SDG or SECO reduced total cholesterol and LDL-cholesterol within normal ranges in rats fed a high cholesterol diet. To understand the underlying mechanism of lignan action in this model system requires a complete understanding of lignan pharmacokinetics. Therefore, the purpose of my M.Sc. project was to conduct a pharmacokinetic analysis of SECO in the rat to enhance our understanding of flaxseed lignan absorption and disposition characteristics. The aims of this project were accomplished by the following objectives:

2.7.2 Objective 1

Isolate and purify secoisolariciresinol diglucoside and secoisolariciresinol from 40% lignan starting material.

2.7.3 Objective 2

Develop and validate an HPLC method with fluorescence detection for quantification of secoisolariciresinol, enterodiol and enterolactone in rat serum.

2.7.4 Objective 3

Assess the pharmacokinetics of secoisolariciresinol after single intravenous and oral bolus administration in rat.

3. MATERIALS AND METHODS

3.1 Isolation of SDG and SECO From 40% Flaxseed Lignan Starting Material

3.1.1 Chemicals

SDG (40% purity) starting material, SECO (≥99% purity), SDG (≥99% purity) were kindly provided by Dr. Muir's lab, Agriculture and Agri-Food Canada Saskatoon Research Center, SK. Water (Optima LC/MS grade), acetonitrile (Optima LC/MS grade), methanol (HPLC grade), glacial acetic acid (certified ACS grade), hydrochloric acid (HCl, certified ACS grade), formic acid (Optima LC/MS grade), sodium hydroxide (NaOH, 40% analytical grade) were purchased from Fisher Scientific Company (Ottawa, ON, Canada). Water for preparative chromatography was purified using a Millipore super Q water system (Millipore, Bedford, MA, USA).

3.1.2 Preparative High Performance Liquid Chromatography (HPLC)

SDG and SECO purification was performed using a Waters PrepLC 4000 system (Waters Corporation, Milford, MA, USA) which included a 486 Tunable Absorbance Detector set at 280nm, PrepLC controller with pumps, and PrepLC Universal Base with 3 (40 $\times$100 mm Waters C_{18} Bondapak 15-20 μm, 125Å packing) cartridges and 1 (40 $\times$100 mm Waters C_{18} Bondapak) guard insert. SDG and SECO were separated under several gradients (Tables 3.1.1-3.1.3) at flow rate of 50 mL/min. Mobile phase consisted of 1% acetic acid or 2% acetic acid in Millipore water and methanol. At 0.05 min into the gradient, the starting material (SDG 40%; SECO 40%, or SECO 75%) in an 80:20 water:methanol mixture was introduced into the preparative HPLC system (Table 3.1, 3.2, and 3.3 respectively).

Table 3.1.1 Preparative HPLC mobile phase linear gradient conditions for secoisolariciresinol diglucoside (SDG) purification

Time (min)	1% Acetic acid in Millipore water (%)	Methanol (%)
0	95	5
0.05	-	-
0.55	95	5
8	90	10
25	60	40
80	10	90
90	95	5
120	95	5

Table 3.1.2 Preparative HPLC mobile phase linear gradient conditions for secoisolariciresinol (SECO) (40%) purification

Time (min)	2% Acetic acid in Millipore water (%)	Methanol (%)
0	95	5
0.05	-	-
0.55	95	5
5	95	5
20	80	20
55	70	30
75	5	95
80	5	95
90	95	5
120	95	5

Table 3.1.3 Preparative HPLC mobile phase linear gradient conditions for secoisolariciresinol (SECO) (75%) purification

Time (min)	2% Acetic acid in Millipore water (%)	Methanol (%)
0	80	20
0.05	-	-
0.55	80	20
15	80	20
55	50	50
75	5	95
85	5	95
95	95	5
120	95	5

3.1.3 Analytical Ultra Performance Liquid Chromatography (UPLC)

SDG and SECO content in fractions collected during preparative HPLC or during the acid hydrolysis (see section 3.1.6) was controlled using a Waters Acquity UPLC System (Waters Corporation, Milford, MA, USA). SDG and SECO were separated on an Acquity UPLC BEH C_{18} column (2.1×50 mm, 1.7 μm particle size, Waters Corporation, Milford, MA, USA) using several gradients (Tables 3.1.4-3.1.5). Mobile phase consisted of 1% formic acid in water and acetonitrile. Injection volume was 15 μL. Analyte peaks were detected at 280 nm using an Acquity Photodiode Array (PDA) detector (Waters Corporation, Milford, MA, USA).

Table 3.1.4 UPLC mobile phase linear gradient conditions for control of secoisolariciresinol diglucoside (SDG) content in fractions collected

Time (min)	Flow rate (mL/min)	1% Formic acid in Optima water (%)	Acetonitrile (%)
0	0.8	95	5
0.25	0.8	95	5
0.6	0.8	80	20
1.95	0.8	30	70
2.25	0.8	95	5
4.25	0.8	95	5

Table 3.1.5 UPLC mobile phase linear gradient conditions for control of secoisolariciresinol (SECO) content

Time (min)	Flow rate (mL/min)	1% Formic acid in Optima water (%)	Acetonitrile (%)
0	0.6	95	5
2.5	0.6	50	50
2.61	0.6	95	5
3.25	0.6	95	5

3.1.4 Ultra Performance Liquid Chromatography/Mass Spectrometry (UPLC/MS)

SDG and SECO identity was confirmed by mass spectrometry using a Synapt HDMS System (Waters Corporation, Milford, MA, USA) with Synapt Q-TOF analyzer at negative ES ionization mode and Waters Acquity UPLC System with Waters Acquity PDA detector set at 280 nm. UPLC analysis was performed using the mobile phase gradient indicated in Table 3.1.4 with 1% formic acid in water and acetonitrile as mobile phase on an Acquity UPLC BEH C_{18} column (2.1×50 mm, 1.7 μm particle size, Waters Corporation, Milford, MA, USA).

3.1.5 Secoisolariciresinol Diglucoside (SDG) Purification

Starting material (SDG content 40%) was dissolved in water-methanol (80:20) mixture and injected onto the preparative column (Table 3.1.1). Time intervals for fraction collection were set in accordance with time of SDG peak elution. Fractions were analyzed for SDG content using UPLC (Table 3.1.4), evaporated to dryness in rotary evaporator (RE-121, Buchi Laboratoriums-Technik AG, Switzerland), dissolved in Millipore water, and freeze-dried (FreeZone Freeze Dryer, Labconco Co., Kansas City, MO, USA). Lyophilisates with SDG content less than 80% were subjected to further purification.

3.1.6 Secoisolariciresinol (SECO) Production and Purification

SDG lyophilisate (22 g, 80% purity fraction obtained in Section 3.1.5) was hydrolyzed in the presence of 1M HCl in 70% methanol at 95°C. The reaction was monitored by injecting neutralized samples onto the UPLC and determining the content of SDG and SECO (Table 3.1.5). The reaction was stopped by adding 50% NaOH to pH=8 when there was no further increase in SECO concentration as indicated by UPLC. Hydrolysis products were further purified and separated on a low-pressure column (C_{18} packing material, 55-110 μm) by washing with a water-methanol mixture with gradual increase of organic content from 5% to 90%. Two fractions of interest were obtained (75% and 40% SECO content), which were further purified on the preparative column as above (Tables 3.1.2-3.1.3). Fractions collected were evaporated to dryness, dissolved in Millipore water, and freeze-dried. In the process of purification SECO concentration was controlled by UPLC (Table 3.1.5).

3.2 Development and Validation of an HPLC Method with Fluorescence Detection for Quantification of Secoisolariciresinol (SECO), Enterolactone (EL) and Enterodiol (ED) in Rat Serum.

3.2.1 Chemicals

HPLC grade enterodiol, enterolactone, and 7-hydroxycoumarin (7-HC) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Secoisolariciresinol was produced and purified by myself in Dr. Muir's lab in Agriculture and Agri-Food Canada, Saskatoon Research Center, SK (≥95% purity, confirmed by UPLC-MS). Acetonitrile HPLC grade was purchased from EMD Chemicals Inc. (Gibbstown, NJ, USA). Water was purified using a MilliQ Synthesis system (Millipore, Bedford, MA, USA). All other chemicals were of analytical grade.

3.2.2 High Performance Liquid Chromatography (HPLC) Conditions

The chromatographic system used for analysis was an Agilent Technologies 1200series (Agilent Technologies Inc., Mississauga, ON, Canada) with a Quaternary Pump (G1311A), Degasser (G1322A), Autosampler (G1329A), TCC (G1316A), and Fluorescence Detector (G1321A). Chromatographic separation was achieved with a Waters Symmetry C_{18} reversed-phase column (4.6×150 mm, 5 μm particle size, Waters Corporation, Milford, MA, USA). The column was kept at 25°C. Wavelengths monitored were 277 nm for excitation and 617 nm for emission. Analytes were separated under gradient mobile phase conditions (Table 3.2.1) with a mobile phase that consisted of 0.1% formic acid in Millipore water and 0.1% formic acid in acetonitrile, at a flow rate of 1 mL/min and injection volume 50 μL. The column was washed with water:methanol (50:50) after every use.

Table 3.2.1 HPLC mobile phase linear gradient conditions for secoisolariciresinol (SECO), enterodiol (ED), and enterolactone (EL) separation

Time (min)	0.1% formic acid in water (%)	0.1% formic acid in acetonitrile (%)
0	85	15
12	50	50
14	10	90
18	10	90
20	85	15
25	85	15

3.2.3 Preparation of Standard Curve Solutions

Primary stock solutions (1 mg/mL) of SECO, ED, EL and 7-HC, as internal standard (IS), were prepared in methanol and stored at -20°C. Mixed working solutions of SECO, ED and EL were prepared by serial dilution of primary stocks with water-acetonitrile (70:30) mixture in the range 0.1-100 µg/mL**.** Working stock solution of 7-HC (100 µg/mL) was prepared from primary stock solution using water-acetonitrile (70:30) mixture as a solvent. Quality control (QC) working solutions presenting the entire range of standard curve: lower limit of quantification (LLOQ), 3-fold the LLOQ low QC sample (LQC), near the center (MQC), and near the upper boundary of standard curve (HQC), were prepared using water-acetonitrile (70:30) mixture as a solvent (Table 3.2.1).

Table 3.2.2 Concentrations of secoisolariciresinol (SECO), enterodiol (ED), and enterolactone (EL) used for preparation of QC working solutions

Compound	LLOQ (ng/mL)	LQC (ng/mL)	MQC (µg/mL)	HQC (µg/mL)
SECO	100	300	40	80
ED	100	300	40	80
EL	500	1500	40	80

To prepare the standard curve, 10 μL of working solutions was added to 90 μL of blank rat serum to achieve calibration standard curve range of 10 ng/mL-10 μg/mL for SECO and ED, and 50 ng/mL-10 μg/mL for EL. QC samples were prepared by spiking 90 μL of blank rat serum with 10 μL of QC working solutions.

3.2.4 Extraction Procedure

To 100 μL of serum, calibration standards or QC samples 10 μL of the working stock of IS, and 4 mL of diethyl ether were added. The mixture was vortexed for 10 min, followed by centrifugation (3200 rpm; 4°C) for 10 min in an Eppendorf centrifuge (Model 5804, Brinkman Instruments Inc., Westbury, NY, USA). The aqueous portion of the centrifuged sample was snap frozen in liquid nitrogen and the organic layer was transferred into disposable 16×100 mm glass tubes and dried down in a CentriVap Concentrator (Labconco Co., Kansas City, MO, USA) under vacuum at 50°C for 30min. Samples were reconstituted in 100 μL of water-acetonitrile (70:30) mixture, vortexed for 1.5 min, transferred into HPLC vials and injected (50 μL) onto the HPLC system.

3.2.5 Method Validation

Method validation was performed according to FDA guidelines[145]. The following validation parameters have been assessed: specificity, linearity and range, limit of detection, limit of quantification, recovery, accuracy and precision.

Method specificity was tested by analyzing six different sources of rat serum to ensure the absence of endogenous compounds with the same retention times as SECO, ED, and EL. A 10-point calibration curve in the range 10 ng/mL-10 μg/mL for SECO and ED, and 50 ng/mL-10 μg/mL for EL (blank and zero samples were also included) was constructed to evaluate linearity of the method. The peak area ratios between analytes and IS were plotted against the nominal concentration of analytes. A linear least-squares regression analysis with weighting ($1/x^2$) was applied to determine slope, intercept and coefficient of determination (r^2).

The accuracy and precision of the method were determined by analysis of the QC samples. The intra-day accuracy and precision were assessed by analysis of six replicates of QC samples (LQC, MQC, and HQC) on a single assay day. The inter-day accuracy and precision were determined from the same QC samples analyzed on three consecutive days (n=18).

Accuracy (%) was expressed as [(actual concentration/expected concentration)*100], and precision was expressed as % relative standard deviation (R.S.D.).

The limit of detection (LOD) was defined as the lowest detectable concentration, taking into consideration a signal-to-noise ratio of 3. The lower limit of quantification (LLOQ) was determined at the lowest concentration at which the precision and accuracy were within 20% of the mean and true value, respectively.

The recovery of analytes was determined by comparison of peak areas obtained by analysis of QC samples prepared in serum followed by extraction procedure and QC samples prepared in mobile phase (n=6).

3.3 Assessment of the Pharmacokinetics (PK) of Secoisolariciresinol (SECO) After Single Intravenous (IV) and Oral Bolus Administration in Rat

3.3.1 Chemicals

Enterodiol (HPLC grade), enterolactone (HPLC grade), 7-hydroxycoumarin (HPLC grade), polyethylene glycol 300 (PEG 300), benzyl alcohol, Tween 80, and ethanol, all analytical grade, β-glucuronidase sulfatase from Helix Pomatia (G1512) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Secoisolariciresinol was produced and purified by myself in Dr. Muir's lab in Agriculture and Agri-Food Canada, Saskatoon Research Center, SK (≥95% purity, confirmed by UPLC-MS). Acetonitrile HPLC grade was purchased from EMD Chemicals Inc. (Gibbstown, NJ, USA). Water was purified using a MilliQ Synthesis system (Millipore, Bedford, MA, USA). All other chemicals were of analytical grade.

3.3.2 Animals

Wistar male rats weighing 260-320g (n=18) were obtained from Charles River Canada (St. Constant, PQ, Canada). For the pilot studies Wistar male rats weighing 350-400g (n=4) were obtained from the Animal Resources Centre of the University of Saskatchewan (Saskatoon, SK, Canada). Animals were housed in groups of two at a controlled temperature and were maintained on a 12 hour dark-light cycle. The animals received a standard laboratory rat chow and water ad libitum throughout the acclimatization period (one week). This work was approved by the Univeristy of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

3.3.3 Secoisolariciresinol (SECO) Dosing Vehicle

The following mixture was used as a dosing vehicle for SECO intravenous and oral administration to rats: polyethylene glycol 300, Tween 80, benzyl alcohol, and ethanol in proportion 65:8:3:24 by volume, considering SECO's poor water solubility. SECO concentration in prepared vehicle was 20 mg/mL.

3.3.4 The 24-hour (Sampling Schedule Optimization) and the 11-hour Pilot Studies (Glucuronidase Pretreatment)

For the 24-hour pilot study a single dose of SECO (40mg/kg) was administered to the rat (n=1) by oral gavage (18 gauge × 3 in). Blood (250 μL) was collected into a plastic microcentrifuge tube (0.65 mL) via saphenous venepuncture at 0 (pre-dose), 15, 30, 45, 60, 90 min, and 2, 3, 7, 11 and 24 hours after the dosing.

For the 11-hour study a single dose of SECO (40 mg/kg) was administered to the rat (n=1) by oral gavage (18 gauge × 3 in). Blood (500μL) was collected into a plastic microcentrifuge tube (0.65 mL) via saphenous venepuncture at 0 (pre-dose), 30 min, 60 min, and 2, 3, 7, and 11 hours after the dosing.

The animals were fasted for 10 hours prior to the dose administration and were given food 3 hours after the dosing. The animals were terminated after completion of the study under isoflurane anaesthesia by opening chest cavity and cutting the left ventricle of the heart.

3.3.5 Rat Jugular and Femoral Vein Cannulation (36-hour Pilot, 12-hour, and 48-hour Studies)

In order to take blood samples and to dose animals, either jugular or jugular and femoral vein cannulation was performed 24 hours prior to the study. Briefly, vascular cannulas (Silastic tubing, I.D. x O.D. (0.64 mm x 1.19 mm), Dow Corning Co., Midland, MI, USA) were implanted into the rat right jugular and left femoral veins under the isoflurane anaesthesia. For the orally dosed animals only the right jugular vein was cannulated. The cannulas were passed subcutaneously to the cannulation site through the skin incision in the dorsal cervical area of the animal. A 500 IU/mL heparin in 0.9% saline was instilled into the cannulas to prevent clotting. Rats were closely monitored to insure their full recovery after the surgery.

3.3.6 The 36-hour Pilot Study

A single dose of SECO (20 mg/kg) was administered via oral gavage (18 gauge × 3 in) or intravenously via a femoral vein cannula (one animal for each route of administration). The animals were fasted for 10 hours prior to the dose administration and were given food 3 hours after the dosing. Blood (250 μL) was drawn through the jugular cannula into a plastic microcentrifuge tube (0.65 mL) at 0 (pre-dose), 5, 10, 15, 20, 30, 45, 60 min, and 2, 4, 8, 12, 18, 24, and 36 hours after the dosing. After each blood sample the same amount of 0.9% saline was injected into the jugular cannula to replace the fluid loss. The animals were terminated after completion of the study under isoflurane anaesthesia by opening chest cavity and cutting the left ventricle of the heart.

3.3.7 The 12-hour Pharmacokinetic Study

A single dose of SECO was administered via oral gavage (18 gauge × 3 in) (40 mg/kg) or intravenously (20 mg/kg) via a femoral vein cannula (six animals for each route of administration). The parallel study design was preferable for the experiment due to the risk of the catheter blockage during the wash-out period between the different routes of administration. The animals were fasted for 10 hours prior to the dose administration and were given food 3 hours after the dosing. Blood (250 μL) was drawn through the jugular cannula into a plastic microcentrifuge tube (0.65 mL) at 0 (pre-dose), 5, 10, 15, 20, 30, 45, 60 min, and 2, 4, 8, and 12 hours after the dosing. After each blood sample the same amount of 0.9% saline was injected into the jugular cannula to replace the fluid loss. The animals were terminated after completion of the study under isoflurane anaesthesia by opening chest cavity and cutting the left ventricle of the heart.

3.3.8 The 48-hour Pharmacokinetic Study

A single dose of SECO was administered via oral gavage (18 gauge × 3 in) (40 mg/kg) or intravenously (20 mg/kg) via a femoral vein cannula (three animals for each route of administration). The parallel study design was preferable for the experiment due to the risk of the catheter blockage during the wash-out period between the different routes of administration. The animals were fasted for 10 hours prior to the dose administration and were given food 3 hours after the dosing. Blood (250 μL) was drawn through the jugular cannula into a plastic microcentrifuge tube (0.65 mL) at 0 (pre-dose), 8, 10, 12, 15, 18, 21, 24, 30, 36, 42, and 48 hours

after the dosing. After each blood sample the same amount of 0.9% saline was injected into the jugular cannula to replace the fluid loss followed by 500 IU/mL heparin in 0.9% saline instillation to prevent clotting. The animals were terminated after completion of the study under isoflurane anaesthesia by opening chest cavity and cutting the left ventricle of the heart.

3.3.9 Serum Preparation

Blood samples were allowed to clot for 30 min at a room temperature and were then centrifuged in an Eppendorf centrifuge (Model 5417C, Brinkman Instruments Inc., Westbury, NY, USA) at 3500 rpm for 5 min. Separated serum was transferred into 0.65 mL plastic microcentrifuge tubes and was stored at -80°C until analysis.

3.3.10 Glucuronidase/Sulfatase Sample Pretreatment for the 11-hour Pilot Study

In order to measure total concentration of SECO, ED, and EL in rat serum after SECO administration, samples were incubated with a freshly prepared mixture of β-glucuronidase sulfatase from Helix Pomatia in 0.5 mol/L (pH 5.0) sodium acetate buffer as reported[113] prior to HPLC analysis. 100 µL of sodium acetate buffer (0.1 mol/L, pH 5.0) and 20 µL of enzyme mixture (100 g/L) were added to 100 µl of serum. The mixture was then incubated in a shaking water bath at 37°C for 4 hours followed by the extraction with diethyl ether as described in Section 3.2.

3.3.11 High Performance Liquid Chromatography (HPLC) Analysis

The HPLC method with fluorescence detection described in Section 3.2 was used for the serum sample analysis. A standard curve was constructed every time prior to analysis. QC samples were analyzed with each analysis run as acceptance criteria for each run.

3.3.12 SECO Pharmacokinetic Parameter Estimation and Statistical Analysis

Non-compartmental methods were used for assessment of SECO PK parameters based on the results of the 12-hour study. GraphPad Prism 4.0 (GraphPad software, San Diego, CA, USA) and Excel were used for calculations. PK parameters are expressed as mean ± SD.

The area under the curve (AUC) and the area under the first moment curve (AUMC) defined by equations 3.3.1 and 3.3.2 were calculated using trapezoidal rule.

$$AUC = \int_0^{\infty} C(t) \times dt \qquad \text{(Equation 3.3.1)}$$

$$AUMC = \int_0^{\infty} t \times C(t) \times dt \,, \qquad \text{(Equation 3.3.2)}$$

where C(t) is SECO serum concentration (ng/mL) at time t (hours).

The mean residence time (MRT) was calculated as ratio of AUMC and AUC (Equation 3.3.3).

$$MRT = \frac{AUMC}{AUC} \qquad \text{(Equation 3.3.3)}$$

Systemic clearance (Cl_S) was determined as dose over AUC following IV SECO administration:

$$Cl_S = \frac{DOSE}{AUC_{IV}} \qquad \text{(Equation 3.3.4)}$$

Elimination rate constant (k) was determined as a slope of the terminal part of the semi-logarithmic plot (SECO concentration versus time after single IV bolus).

An apparent volume of distribution at steady state (V_{SS}) was determined as product of systemic clearance and MRT after SECO single IV bolus:

$$V_{SS} = Cl_S \times MRT_{IV} \qquad \text{(Equation 3.3.5)}$$

Volume of distribution was also determined using following equation:

$$V_d = \frac{DOSE}{k \times AUC_{IV}} \qquad \text{(Equation 3.3.6)}$$

SECO half-life ($t_{1/2}$) was determined as follows:

$$t_{1/2} = 0.693 \times MRT_{IV} \qquad \text{(Equation 3.3.7)}$$

Alternatively SECO half-life ($t_{1/2}$) was determined as follows:

$$t_{1/2} = \frac{0.693}{k} \qquad \text{(Equation 3.3.8)}$$

SECO oral bioavailability (F_{ORAL}) was calculated using the following equation:

$$F_{ORAL} = \frac{AUC_{ORAL} \times DOSE_{IV}}{AUC_{IV} \times DOSE_{ORAL}} \,, \qquad \text{(Equation 3.3.9)}$$

where AUC_{IV} is the mean of results for the animals dosed intravenously (n=6) and AUC_{ORAL} is the mean of results for the animals dosed orally (n=6), $DOSE_{IV}$ is 20 mg/kg and $DOSE_{ORAL}$ is 40 mg/kg.

4. RESULTS

4.1 Isolation of SDG and SECO From 40% Flaxseed Lignan Starting Material

The preparative process yielded 15 g of SDG (≥95% purity) from the starting material containing 40% of SDG (140g). The purity of compound was determined by UPLC analysis on the basis of the area percentage of the peak absorbed at 280 nm (Figure 4.1.1).

SECO was produced from SDG by acid hydrolysis. SDG was firstly converted into secoisolariciresinol monoglucoside (SMG), which was then hydrolyzed to SECO. It was important to monitor concentration of SECO in the reaction mixture as SECO molecule is not stable under the applied conditions and could be transformed to its anhydrous form anhydrosecoisolariciresinol (ASECO), depending on the duration of hydrolysis and acid concentration. Final concentrations of the hydrolysis products based on the area percentage of peaks absorbed at 280 nm were SDG 3.5%, SMG 23%, SECO 23%, ASECO 30% (identity of compounds was confirmed by comparison with chromatograms of authentic standards).

The acid hydrolysis procedure yielded 1.83 g of SECO (≥95% purity) from 22 g of SDG (80% purity) starting material. The purity of SECO was determined by UPLC analysis on the basis of the area percentage of the peak absorbed at 280 nm (Figure 4.1.2).

Identity of purified SDG and SECO was also confirmed by quadrupole time-of-flight mass spectrometry in negative ionization mode which exhibited ions at *m/z* 685.2 and 361.1, corresponding to $[M-H]^-$ ions for SDG and SECO.

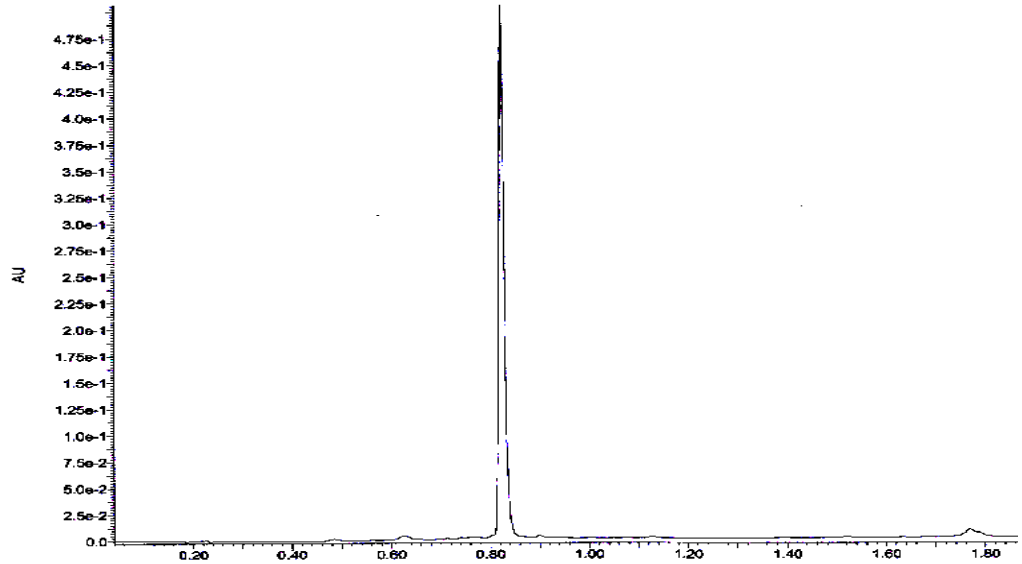


Figure 4.1.1 Representative UPLC chromatogram of purified SDG (absorbance at 280 nm) with retention time 0.83 min (UPLC chromatographic conditions are indicated in Section 3.1).

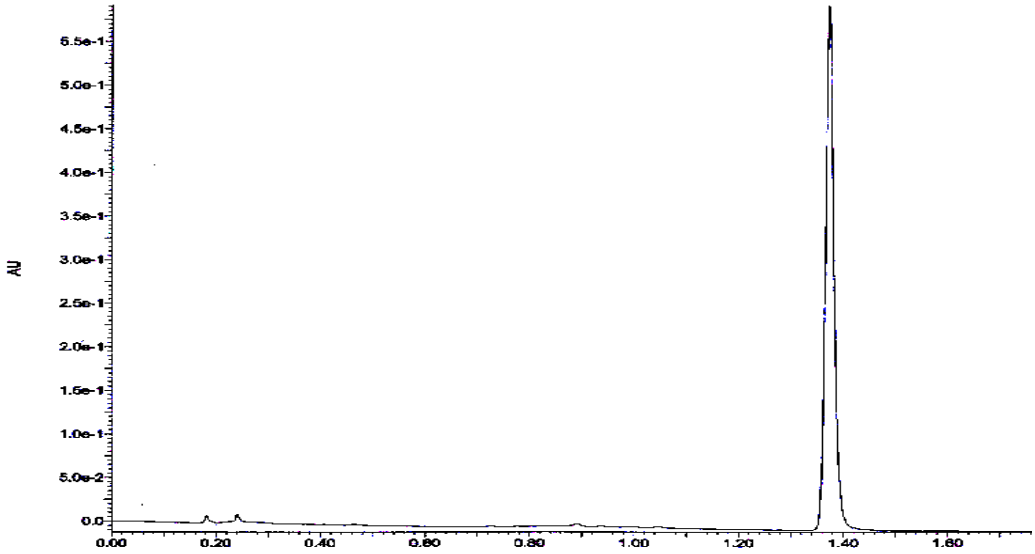


Figure 4.1.2 Representative UPLC chromatogram of purified SECO (absorbance at 280 nm) with retention time 1.38 min (UPLC chromatographic conditions are indicated in Section 3.1).

4.2 Development and Validation of an HPLC Method with Fluorescence Detection for Quantification of Secoisolariciresinol (SECO), Enterolactone (EL) and Enterodiol (ED) in Rat Serum.

Typical chromatograms of blank serum and serum spiked with working solutions of SECO, ED, EL, and IS demonstrate that there are no endogenous peaks that could interfere with analytes and the method is specific under the specified chromatographic conditions. The retention times for IS, SECO, ED, and EL are 7.0, 7.9, 9.4, and 12.3 min, respectively (Figure 4.2.1).

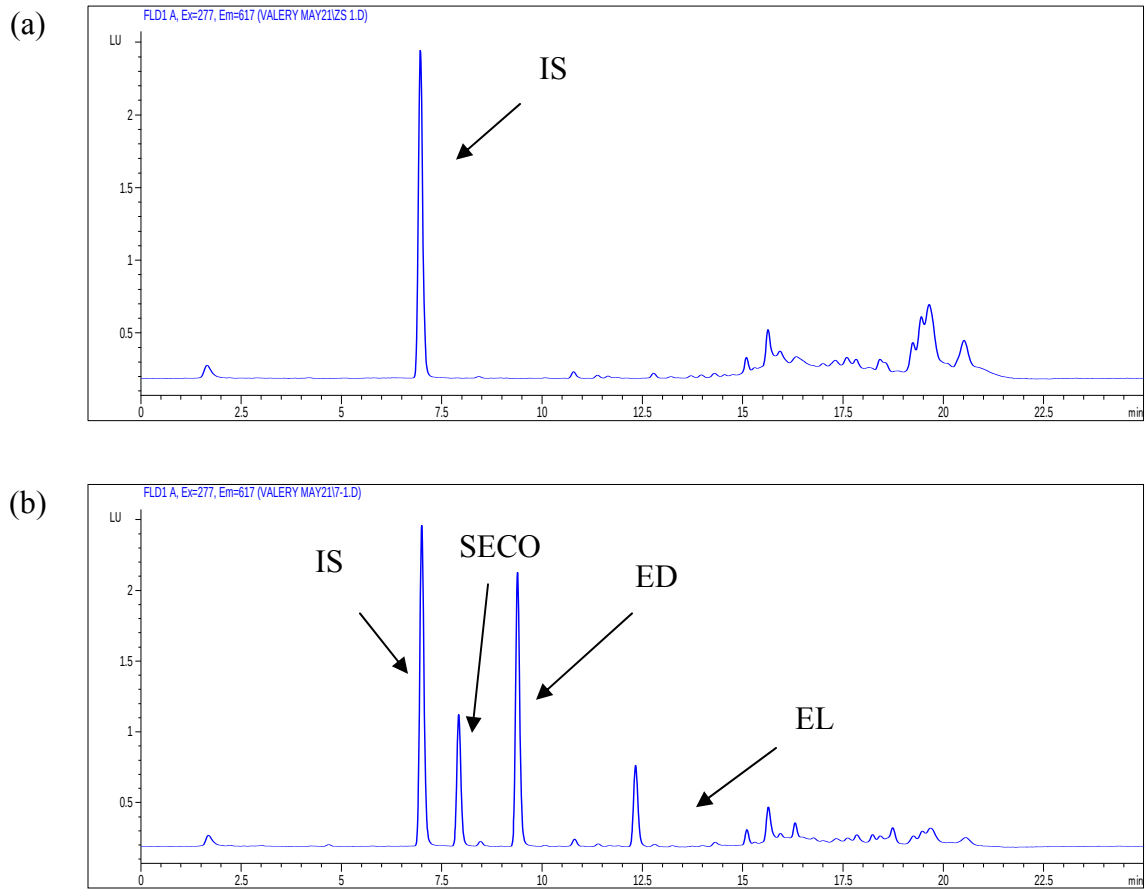


Figure 4.2.1 Representative HPLC chromatograms: (a) blank rat serum spiked with internal standard; (b) blank rat serum spiked with mixed working solution (1 µg/mL) of SECO (7.9 min), ED (9.4 min), EL (12.3 min) and internal standard (7.0 min).

The LOD for SECO, ED, and EL are 3 ng/mL, 3 ng/mL, and 12 ng/mL, respectively. The LLOQ was determined to be 10 ng/mL (SECO), 10 ng/mL (ED), 50 ng/mL (EL) with acceptable accuracy (80-120%) and precision (R.S.D. < 20%). The calibration curves of the peak area ratios versus analytes concentration showed good linear responses over a wide range of concentrations (10 ng/mL-10 μg/mL). Coefficients of determination were greater than 0.99, relevant slope values were statistically different from 0 ($P<0.001$), while intercepts were not significantly different from zero (Student's t-test, $P<0.05$, Prism 4.0, GraphPad software, San Diego, CA, USA) (Table 4.2.1).

Table 4.2.1 Linearity data for the determination of SECO, ED, and EL with the proposed HPLC method

SECO calibration curve			ED calibration curve			EL calibration curve		
slope	intercept	r^2	slope	intercept	r^2	slope	intercept	r^2
0.000460	-0.00027	0.996	0.001002	-0.00119	0.999	0.000218	-0.00183	0.997
0.000469	-0.00063	0.993	0.001015	0.00119	0.997	0.000207	-0.00144	0.995
0.000445	-0.00049	0.990	0.000974	-0.00002	0.999	0.000191	0.00033	0.996
mean								
0.000458	-0.00046	0.993	0.000997	-0.000004	0.998	0.000205	-0.00098	0.996
S.D.								
0.00001	0.00010	0.002	0.00001	0.000689	0.001	0.000008	0.00066	0.001

S.D.: standard deviation of the mean

During the method validation intra- and inter-day precisions were less than 10% and the accuracy of estimated analytes concentrations ranged from 93 to 110% (Tables 4.2.2-4.2.4). The mean (±SD) recoveries of SECO at 30 ng/mL, 4 μg/mL, and 8 μg/mL were 106±6.8%, 105±0.5%, and 108±1.1%, respectively; of ED at 30 ng/mL, 4 μg/mL, and 8 μg/mL were 108±4.7%, 105±0.7%, and 109±1.2%, respectively; of EL at 150 ng/mL, 4 μg/mL, and 8 μg/mL were 102±3.9%, 98.7±0.7%, and 102±1.3%, respectively.

Table 4.2.2 Intra-day (n=6) and inter-day (three consecutive days) accuracy and precision values of SECO determination by proposed HPLC method in rat serum

SECO nominal concentration in QCs (μg/mL)	Intra-day accuracy and precision			Inter-day accuracy and precision		
	Observed concentration (mean±S.D., μg/mL)	Accuracy (%)	Precision (CV%)	Observed concentration (mean±S.D., μg/mL)	Accuracy (%)	Precision (CV%)
0.03	0.030±0.002	100.3	7.1	0.030±0.001	101	5.48
4	4.31±0.14	107.6	3.16	4.29±0.11	107	2.57
8	7.87±0.38	98.3	4.83	7.88±0.47	98.5	5.99

Table 4.2.3 Intra-day (n=6) and inter-day (three consecutive days) accuracy and precision values of ED determination by proposed HPLC method in rat serum

ED nominal concentration in QCs (μg/mL)	Intra-day accuracy and precision			Inter-day accuracy and precision		
	Observed concentration (mean±S.D., μg/mL)	Accuracy (%)	Precision (CV%)	Observed concentration (mean±S.D., μg/mL)	Accuracy (%)	Precision (CV%)
0.03	0.032±0.0006	105.2	1.93	0.031±0.0008	106.2	2.43
4	4.16±0.18	104	4.33	4.08±0.14	101.9	3.41
8	8.43±0.216	105.3	2.57	8.40±0.40	105	4.72

Table 4.2.4 Intra-day (n=6) and inter-day (three consecutive days) accuracy and precision values of EL determination by proposed HPLC method in rat serum

EL nominal concentration in QCs (μg/mL)	Intra-day accuracy and precision			Inter-day accuracy and precision		
	Observed concentration (mean±S.D., μg/mL)	Accuracy (%)	Precision (CV%)	Observed concentration (mean±S.D., μg/mL)	Accuracy (%)	Precision (CV%)
0.15	0.147±0.009	98.2	6.43	0.152±0.008	101.7	5.66
4	3.90±0.28	97.6	7.11	3.99±0.24	99.9	6.11
8	8.37±0.35	104.5	4.72	8.58±0.40	107.2	4.63

Collectively the data suggest that the new gradient reversed-phase HPLC method with fluorescence detection is suitable for the simultaneous determination of lignans in rat serum. The analytical method is specific, sensitive, accurate and precise for the quantification of SECO, ED, and EL. The total analytical run time is 25 min, which allows us to analyze multiple samples in a relatively short time period. The method is suitable for pharmacokinetic studies which require both sensitive (to detect and quantify small changes in drug concentration over time) and simple (high number of samples to be processed) analytical techniques.

4.3 Assessment of the Pharmacokinetics (PK) of Secoisolariciresinol (SECO) After Single Intravenous (IV) and Oral Bolus Administration in Rat

4.3.1 The 24-hour (Sampling Schedule Optimization) and the 11-hour Pilot Studies (Glucuronidase Pretreatment)

A 24-hour pilot study was performed to optimize blood sampling schedule. The maximum SECO concentration (309.4 ng/mL) was observed 15 min after the oral administration (40 mg/kg) with gradual decrease to 46 ng/mL 24 hours after the dosing. ED was detected in the 24-hour sample, while EL was not detected.

The 11-hour pilot study was performed to compare total and free lignans after SECO single oral dose administration (40 mg/kg). 100 μL of serum at each time point was purified and subjected to HPLC analysis as described in Section 3.2 to monitor free lignan concentration in

rat serum over time. The other portion of serum of the same time point sample was incubated with glucuronidase before the HPLC analysis to hydrolyze lignan conjugates and to observe changes in total lignan concentration over time.

The first spike of free SECO concentration in serum was observed in 30-min sample (346.5 ng/mL) with two additional peaks at 2 hours (320.5 ng/mL) and 11 hours (620 ng/mL) after the dose administration. ED was detected (levels were below the limit of quantification) in 7 and 11 hour samples.

The HPLC analysis of the pre-dose 0 min sample after treatment with glucuronidase showed significant peaks at 7.9 min and 12.3 min, time corresponding to SECO and EL elution, which could be a result of deconjugated compounds with similar retention times from rat diet and should be confirmed by other methods of analysis.

4.3.2 The 36-hour Pilot Study

Taking into consideration results of the 24-hour study, a 36-hour pilot study was performed to further optimize blood sampling schedule after IV and oral SECO administration to allow for the appropriate detection of ED and EL.

The maximum serum SECO concentration (5180 ng/mL) was observed 5 min after the IV SECO administration (20 mg/kg). The serum SECO levels dropped drastically to 276 ng/mL after 1 hour, and to 11 ng/mL at the end of experiment. ED was detected starting from the 12-hour sample and was quantified in 18-hour (15.5 ng/mL) and 24-hour (12.3 ng/mL) samples. EL was detected only at 18 and 24 hours after SECO IV administration.

The maximum serum SECO concentration (181.9 ng/mL) was observed 5 min after the oral SECO administration (20 mg/kg). SECO serum concentration dropped to 11.7 ng/mL at 4 hours after the dose and was not detected in later samples. ED was detected only in the 8- and 12-hour samples. EL was not detected in the rat serum after SECO oral administration.

4.3.3 The 12-hour Pharmacokinetic Study

A 12-hour study was performed to assess SECO concentration profile, based on the results of pilot studies. SECO serum concentration dropped quickly during the first hour after IV administration and by 12 hours concentrations were near the LOQ of the assay. ED was detected (levels were below the LOQ of the assay) at 12 hours after IV administration in rats 1, 2, and 4;

and in rats 1, 3, 6 after oral administration. EL was not detected. Tables 4.3.1 and 4.3.2 summarize results of the 12-hour study.

Table 4.3.1 Serum concentration versus time profile of SECO after single IV bolus (20 mg/kg) to male Wistar rats (n=6)

Time (min)	Rat 1 (ng/mL)	Rat 2 (ng/mL)	Rat 3 (ng/mL)	Rat 4 (ng/mL)	Rat 5 (ng/mL)	Rat 6 (ng/mL)
0	0	0	0	0	0	0
5	4489.83	4156.7	9047.51	5313.25	4666.3	5864.07
10	1714.89	2321.08	5014.24	3177.41	2353.68	3180.65
15	1304.91	1223.61	2643.07	1651.05	1616.75	1626.48
20	855.3	885.37	1827.32	1620.21	955.8	N/A
30	408.76	525.76	921.82	654.84	410.11	420.27
45	183.71	219.56	345.52	259.7	221.55	235.39
60	186.35	165.01	123.13	214.11	138.34	185.72
120	186.98	130.19	35.26	137.69	147.09	141.47
240	D	117.01	17.48	78.3	52.2	D
480	N/D	58.93	78.71	75.81	N/D	N/D
720	N/D	N/D	24.19	D	N/D	30.08

D – detected (below the LOQ of the assay)
N/D – not detected

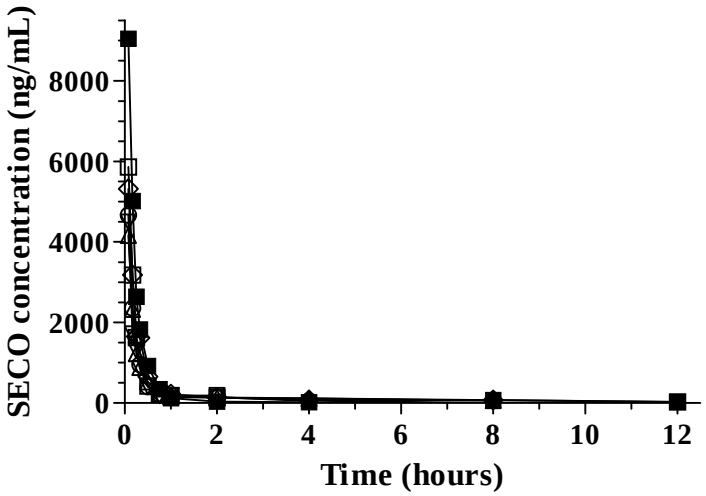
Table 4.3.2 Serum concentration versus time profile of SECO after single oral dose (40 mg/kg) to male Wistar rats (n=6)

Time (min)	Rat 1 (ng/mL)	Rat 2 (ng/mL)	Rat 3 (ng/mL)	Rat 4 (ng/mL)	Rat 5 (ng/mL)	Rat 6 (ng/mL)
0	0	0	0	0	0	0
5	124.23	448.96	315.23	97.35	119.2	40.38
10	216.63	240.56	655.55	90.26	651.38	102.93
15	189.62	163.63	1487.26	67.08	811.57	43.38
20	166.75	109.28	565.56	42.97	N/A	N/A
30	187.99	54.4	301.38	59.63	398.78	37.38
45	297.03	146.24	195.68	102.49	244.04	50.2
60	355.94	140.34	1040.28	152.9	238.37	63.98
120	106.18	81.39	52.71	82.99	74.14	87.84
240	44.07	46.46	102.54	101.57	11.01	D
480	97.21	105.7	73.05	93.15	D	48.49
720	17.14	N/A	150.04	88.38	13.45	30.08

D – detected (below the LOQ of the assay)
N/D – not detected
N/A – not available

Figure 4.3.1 suggests that SECO exhibits two compartment model characteristics with an extensive distribution phase. SECO concentration profile after oral administration is shown on Figure 4.3.2.

(a)



(b)

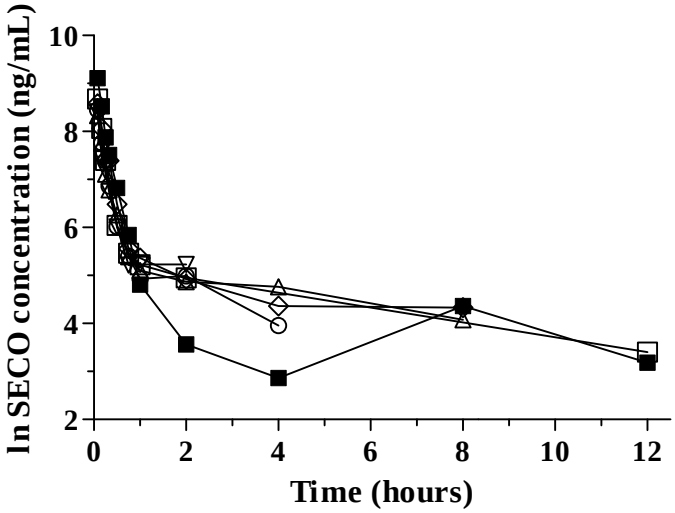
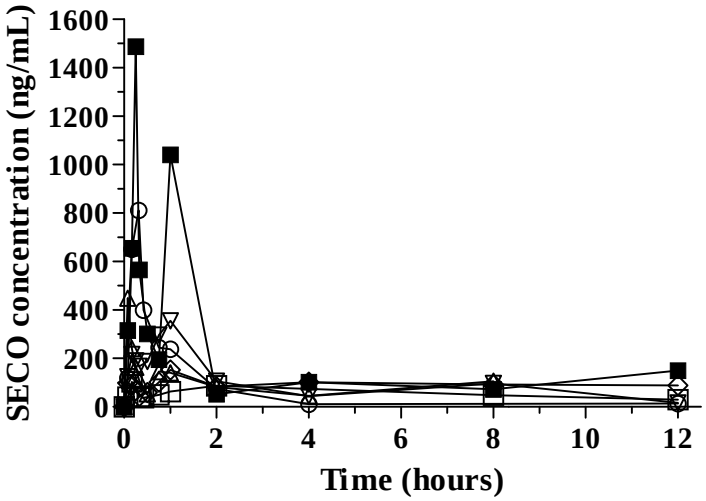


Figure 4.3.1 SECO serum concentration profile after single IV dose (20mg/kg) in six male Wistar rats: (a) rectilinear plot; (b) semilogarithmic plot (rat 1, rat 2, rat 3, rat 4, rat 5, rat 6).

(a)



(b)

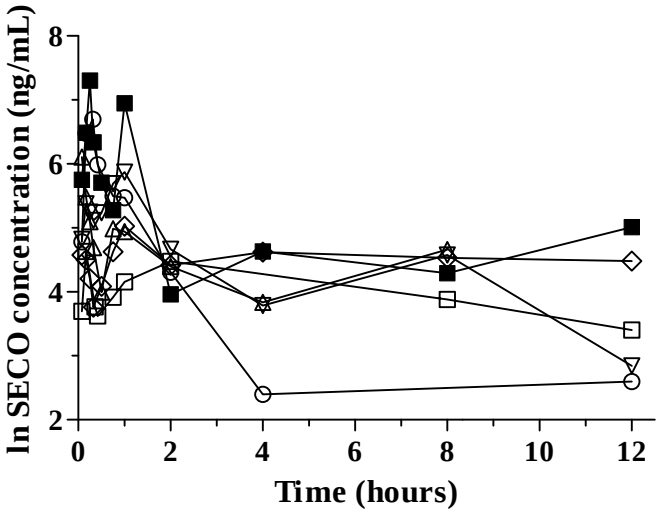


Figure 4.3.2 SECO serum concentration profile after single oral dose (40mg/kg) in six male Wistar rats: (a) rectilinear plot; (b) semilogarithmic plot (rat 1, rat 2, rat 3, rat 4, rat 5, rat 6).

4.3.4 The 48-hour Pharmacokinetic Study

A 48-hour study was conducted to assess SECO, ED and EL concentration profile, based on the results of pilot studies indicating that ED is detectable starting 12 hours after the SECO administration and EL is detectable starting 18 hours after the SECO administration. Tables 4.3.3 and 4.3.4 summarize results of the 48-hour study.

Table 4.3.3 Serum concentration versus time profile of SECO after single oral (40 mg/kg by gastric gavage) and IV (20 mg/kg via femoral cannula) in male Wistar rats (n=3 per dosing route)

Time (hours)	Rat 1 IV (ng/mL)	Rat 2 IV (ng/mL)	Rat 3 IV (ng/mL)	Rat 4 oral (ng/mL)	Rat 5 oral (ng/mL)	Rat 6 oral (ng/mL)
0	0	0	0	0	0	0
8	114.47	66.82	97.18	111.47	detect	67.31
10	86.8	92.41	88.5	66.64	13.87	69.35
12	38.85	55.1	50.63	68.99	22.19	82.81
15	27.58	49.02	22.84	50.64	221.84	23.76
18	D	D	N/D	21.15	125.67	71.19
21	D	D	D	D	107.43	19.37
24	N/D	N/D	D	N/D	124.55	D
30	N/D	N/D	N/D	N/D	45.81	N/D
36	N/D	N/D	N/D	N/D	46.1	N/D
42	N/D	N/D	N/D	N/D	D	N/D
48	N/D	N/D	N/D	N/D	N/D	N/D

D – detected (below the LOQ of the assay)
N/D – not detected

Table 4.3.4 Serum concentration versus time profile of ED after single oral (SECO, 40 mg/kg by gastric gavage) and IV (SECO, 20 mg/kg via femoral cannula) in male Wistar rats (n=3 per dosing route)

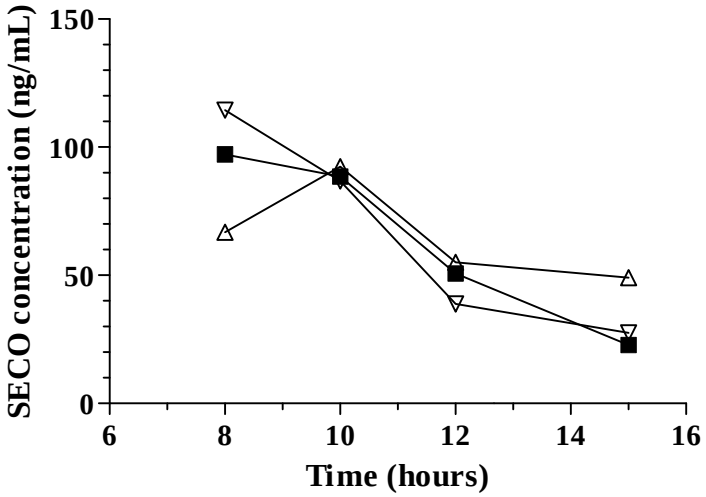
Time (hours)	Rat 1 IV (ng/mL)	Rat 2 IV (ng/mL)	Rat 3 IV (ng/mL)	Rat 4 oral (ng/mL)	Rat 5 oral (ng/mL)	Rat 6 oral (ng/mL)
0	0	0	0	0	0	0
8	D	N/D	D	N/D	N/D	D
10	D	D	D	D	D	N/D
12	N/D	N/D	D	11.48	N/D	N/D
15	D	D	D	D	N/D	D
18	D	D	D	D	D	D
21	11.94	13.28	14.48	D	D	D
24	D	12.15	D	D	N/D	D
30	N/D	N/D	N/D	N/D	N/D	D
36	N/D	N/D	N/D	N/D	D	N/D
42	N/D	N/D	N/D	N/D	D	N/D
48	N/D	N/D	N/D	N/D	D	N/D

D – detected (below the LOQ of the assay)
N/D – not detected

EL was detected in 15, 18, and 36 hours samples of rat 1 (IV dose). It was also detected in 21 and 36 hours sample of rat 4 (oral dose).

SECO concentration versus time is also depicted in Figure 4.3.3.

(a)



(b)

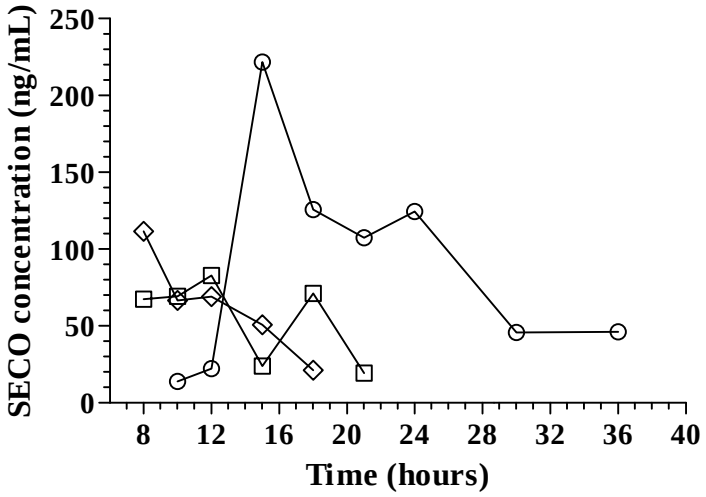


Figure 4.3.3 SECO serum concentration profile in male Wistar rats after: (a) IV bolus (20 mg/kg, n=3); (b) oral administration (40 mg/kg, n=3) (rat 1, rat 2, rat 3, rat 4, rat 5, rat 6).

4.3.5 SECO Pharmacokinetic Parameter Estimation

Non-compartmental methods were used for assessment of SECO PK parameters based on the results of the 12-hour study.

Tables 4.3.5 and 4.3.6 summarize SECO PK parameters calculated using mean residence time and elimination rate constant.

Table 4.3.5 SECO PK parameter estimation based on the results of 12-hour study after single IV (20 mg/kg) or oral (40 mg/kg) dose of SECO (MRT used for determination V_{ss} and $t_{1/2}$)

Route	IV							Oral	
of admin	Weight (g)	AUC_{IV} (ng×h/mL)	$AUMC_{IV}$ (ng×h^2/mL)	MRT_{IV} (h)	Cl_S (L/h)	V_{SS} (L)	$t_{1/2}$ (h)	Weight (g)	AUC_{ORAL} (ng×h/mL)
Rat 1	315	1298	498	0.38	4.85	1.86	0.27	280	1116
Rat 2	310	1870	3060	1.64	3.31	5.42	1.13	310	693
Rat 3	275	2856	3916	1.37	1.93	2.64	0.95	320	2016
Rat 4	300	2246	2999	1.34	2.67	3.56	0.92	260	1139
Rat 5	260	1556	959	0.62	3.34	2.06	0.43	285	714
Rat 6	285	2517	3684	1.46	2.26	3.31	1.01	280	695
Mean		2057			3.06	3.15	0.79		1062
S.D.		591			1.04	1.30	0.35		512

S.D.: Standard deviation

Table 4.3.6 SECO PK parameter estimation based on the results of 12-hour study after single IV (20 mg/kg) or oral (40 mg/kg) dose of SECO (k used for determination of V_d and $t_{1/2}$)

Route of admin	IV						Oral	
	Weight (g)	AUC_{IV} (ng×h/mL)	k (h^{-1})	Cl_S (L/h)	V_d (L)	$t_{1/2}$ (h)	Weight (g)	AUC_{ORAL} (ng×h/mL)
Rat 1	315	1298	0.33	4.85	14.50	2.07	280	1116
Rat 2	310	1870	0.16	3.31	20.92	4.37	310	693
Rat 3	275	2856	0.06	1.93	32.57	11.72	320	2016
Rat 4	300	2246	0.16	2.67	16.43	4.26	260	1139
Rat 5	260	1556	0.39	3.34	8.53	1.76	285	714
Rat 6	285	2517	0.17	2.26	13.30	4.07	280	695
Mean		2057	0.21	3.06	17.71	4.71		1062
S.D.		591	0.12	1.04	8.33	3.62		512

S.D.: Standard deviation

Visual analysis of the semilogarithmic plot of SECO concentration versus time after single IV bolus suggests that calculation of volume of distribution and half-life using elimination rate constant will give more accurate results than using MRT. Mean Residence Time is a ratio of AUMC and AUC from time zero to infinity. As there are not enough points in the tail region of the SECO concentration versus time curve, computational error in estimation of the tail region (to time infinity) when calculating AUC and especially AUMC becomes high.

SECO PK parameters after single IV bolus are as follows: Cl_s=3.06±1.04 L/h, k=0.21±0.12 h^{-1}, V_d=17.71±8.33 L, $t_{1/2}$=4.71±3.62 h, and F_{oral}=26 %.

5. DISCUSSION

5.1 Isolation of SDG and SECO From 40% Flaxseed Lignan Starting Material

The first objective of my research project was isolation of pure SDG and, subsequently SECO, as the latter is not readily available on the market in quantities sufficient for the pharmacokinetic study. Utilization of pure analyte is essential for pharmacokinetic evaluations, as coadministered substances might affect the rate of absorption, distribution and metabolism of the compound being analyzed. For naturally occurring substances, the effect of a biological matrix needs to be taken into consideration as well. For example, the degree to which flaxseed is crushed will affect the biovailability of ED and EL[89]. In healthy volunteers consuming the same amount of whole, crushed, or ground flaxseed, the mean relative bioavailability of ED and EL was only 28% from whole flaxseed and 43% from crushed flaxseed compared to ground flaxseed[89]. As well, high oil component of flaxseed might interfere with lignan absorption.

The metabolic profile of the pure compound could be different from that in the dietary source. Flaxseed contains SDG in the form of lignan polymer (Section 2.2). It takes several hours before SDG is released from the macromolecule, deglycosylated to SECO and absorbed[85]. The process supposedly occurs in the ascending colon skipping the major absorption site, the small intestine[146]. Similarly, in healthy volunteers consuming sesame seeds, which contain both, SDG and SECO, maximum SECO concentration was achieved approximately 6 hours after oral administration[121]. On the other hand, pure SECO administered oraly, could be readily absorbed in the small intestine and would reach the systemic circulation faster (Section 4.3).

A difference in urinary lignan excretion was also observed after a single oral administration of equimolar quantities of SDG and SECO in rats[147]. The 24-hour total lignan (the sum of SECO, ED and EL) urinary excretion after SECO administration was three-fold higher than following the SDG dose. EL and ED were the major lignans excreted after SECO administration, while mostly EL was excreted after SDG administration[147]. Chronic exposure to SECO also results in a drastic increase in EL urinary excretion, with ED being a minor metabolite in rat urine[114].

Although some PK studies were conducted with dietary source of lignans[120,121], the method used to quantify SECO in food samples is another major factor that will affect the study outcome. As was reviewed in Section 2.6, flaxseed and other food samples need hydrolysis, before SECO could be quantified. All hydrolysis methods reported in the literature give up to 26-fold variation in SECO concentration making calculation of the equivalent dose of the dietary

source of SECO very difficult[122]. Moreover, different food matrices require additional experiments for the optimization of the hydrolysis time[140]. Formation of artifacts during hydrolysis is another drawback of some methods of lignan analysis[140]. Therefore, caution should be exercised when interpreting results of such studies. Given these limitations I endeavoured to generate a highly pure source of SECO to ensure reliable PK evaluations.

SECO was produced from SDG by acid hydrolysis with subsequent purification using preparative HPLC. During the reaction, SDG is firstly hydrolyzed to SMG and then SECO is formed. Unfortunately, with prolonged hydrolysis time, SECO breaks down to ASECO and its concentration in the reaction mixture starts to decrease[3]. Therefore, it was very important to monitor changes in SECO concentration during the process, and I had to stop the reaction at a maximum SECO content of 23%. After separation on the low-pressure column, two fractions of interest were chosen for further purification (SECO 75% and SECO 40%). Some difficulties were experienced during the purification of material with 40% SECO content, as it also contained considerable amount of SMG (55%). Due to similar physico-chemical properties SMG and SECO eluted very close together, which required extensive experimenting with gradient conditions to achieve better separation. Moreover, fluctuation of SECO retention time caused by the changes in the room temperature during the preparative HPLC, interfered with collection fractions with maximum SECO content. All above mentioned factors contributed to the low yield of pure SECO (10% of utilized SDG). The final purity of SECO was above 95% and the amounts generated were sufficient to carry out the necessary PK evaluations.

5.2 Development and Validation of an HPLC Method with Fluorescence Detection for Quantification of Secoisolariciresinol (SECO), Enterolactone (EL) and Enterodiol (ED) in Rat Serum.

The second objective of my research was to develop and validate a method for lignan quantification suitable for pharmacokinetic applications. Although mass spectrometry offers high analytical sensitivity, it is not readily available in many laboratories. On the other hand, HPLC with UV detection commonly used for plant lignan samples lacks sensitivity when adapted to biological fluids[138,143,144]. Taking into consideration that lignans show high fluorescence intensity[148,149], development of an HPLC method with fluorescence detection would give an advantage of higher sensitivity, necessary for PK studies. As few endogenous compounds of biological matrices have the ability to fluoresce, the method would be more specific as well.

The developed method is suitable for quantification of SECO, ED, and EL in rat serum. While most of the reported methods determine ED and EL only[95,125,131,134], as was reviewed in Section 2.5, only recently has SECO been included in the serum analysis[129,130]. Low serum volume (100 μL) requirements allow for serial blood sampling necessary for pharmacokinetic applications in small species like the rat. The extraction procedure used for the sample clean-up is relatively straightforward and simple, compared to ion-exchange chromatography and following derivatization in commonly used GC-MS methodology. To account for losses of the analytes during sample purification and evaporation of the solvent during analysis, 7-hydroxycoumarin is used as an internal standard. On the other hand, most MS methods use deuterated or multiply ^{13}C-labeled lignans which are not commercially available and need to be additionally synthetized[115,127,128,132,133]. Moreover, deuterated standards are often unstable and can loose deuterium while in solution. Deuterium could be replaced by hydrogen, which will result in a reduced amount of internal standard used for the sample processing and overestimation of the analyte[128].

A relatively short run time (25 min) of the developed method allows one to process more samples as opposed to some recently published methods which require up to 85 min for analysis[129,130,148]. The developed method does not have the high sensitivity of some HPLC methods utilizing coulometric electrode array detection, however, the accuracy and precision of these methods was reported to be low for SECO (66% accuracy, 37% presicion) and ED (69% accuracy, 44% precision) making them semiquantitave at the lower range of concentrations [117,129].

The developed HPLC method is specific, sensitive, accurate and precise for the quantification of SECO, ED, and EL in rat serum. The simple sample preparation and short run time lowers the costs associated with sample processing and allows handling a number of samples in a relatively short time period. Simultaneous quantification of all SDG metabolites makes the method suitable for a complete pharmacokinetic analysis of lignans in the rat and possible transfer to other matrices and species.

5.3 Assessment of the Pharmacokinetics (PK) of Secoisolariciresinol (SECO) After Single Intravenous (IV) and Oral Bolus Administration in Rat

The third objective of my research was to assess the pharmacokinetics of SECO after a single intravenous and oral bolus administration in rat. Pharmacokinetic characterization of the compound helps identify the relationship between exposure to the compound and observed effect, which is critical in drug design and discovery process. To date, limited PK data exist for flaxseed lignans and SECO, especially, as it was assumed that only enterolignans are responsible for reported health benefits and SECO is a minor metabolite of SDG[3]. On the other hand, recent studies suggest that SECO is found in detectable quantities in plasma of many species, giving a new prospective on which lignan form elicits the observed health benefits [94,121]. Taking into consideration that many studies on biological activity were conducted with a dietary source of lignans as opposed to pure compounds, little research was done regarding potential contribution of SECO to observed health effects.

5.3.1 The 12-hour Pharmacokinetic Study

The aim of this experiment was to study the time course of SECO concentration in the rat after intravascular (intravenous) and extravascular (oral) administration and determine SECO pharmacokinetic parameters. Two pilot studies preceeded the experiment to find the sampling schedule appropriate for the most accurate monitoring of SECO, as it was found to be absorbed very fast. Unfortunately, selected time points did not allow for simultaneous quantification of ED and EL. Results of pilot studies showed that ED was detected in 8 hours, while EL was detected in18 hours after the dose administration. Therefore, more time points were required at the interval between 8 and 24 hours, which was not possible without causing distress in animals due to excessive blood loss. For animal welfare reasons it was decided to conduct a separate 48-hour experiment to monitor changes in ED and EL levels.

Large interindividual variation in SECO serum levels was observed following both routes of administration, which is consistent with other lignan experiments[111,112,120]. SECO concentration declined rapidly during the first hour after IV injection and by the end of experiment was below the LOQ of the assay in four out of six rats, while in two animals SECO was present in somewhat significant quantities. The lignan showed two-compartment model characteristics with an extensive distribution phase.

Following oral administration, two distinct spikes in SECO concentration were observed in the intervals 5-15 min and 45-60 min after the dose. A third spike was observed in three animals at 8 or 12 hours post dose, while another two showed a plateau between 4 and 12 hours. The second peak of SECO serum levels may be explained by disruption in gastric emptying caused by short-term isoflurane anaesthesia which was used to avoid distress in the animals during the oral gavage. Brief isoflurane anaesthesia was found to significantly reduce gastric emptying and gastrointestinal motility in rats measured by charcoal technique[150]. During the experiment, rats were gavaged with gelatin capsule filled with activated charcoal.The effect of anaesthesia on gastric emptying of liquids was not assessed[150]. It is possible that due to delayed gastric emptying initial absorption occurred in the stomach while a second peak was the result of SECO absorption from the intestine.

Another possible explanation for the second spike in SECO serum levels is that it undergoes enterohepatic recirculation. Under fasted conditions gastric emptying of the liquid marker in rats occurs much faster than in fed state and results in early and high serum levels of coadministered drugs[151,152]. For example, an early peak plasma level was observed after an oral gavage of diclofenac in rats[153]. The study investigated diclofenac pharmacokinetics in rats, including enterohepatic recirculation using normal and bile duct-cannulated rats. After intraduodenal administration of diclofenac, plasma levels in normal animals showed a plateau 1 hour after the dose, while in the bile-duct cannulated animals, diclofenac plasma concentration continued to decline[153]. Some anatomical differences such as constant bile flow and elevated β-glucuronidase activity in the proximal small intestine compared to humans may also shorten time for lignans to be excreted into the duodenum, hydrolyzed and then reabsorbed[146,154]. On the other hand, a pharmacokinetic study with structurally similar resveratol using linked-rat model showed a plasma peak attributed to enterohepatic recirculation in bile-recipient rats 6 hours after the oral dose, which is consistent with third spike of SECO levels in my study[155]. The very high early peak of SECO is somewhat unusual for orally administered compounds. Although it is possible that some absorption have occurred in the stomach, the surface area of the stomach mucosa is much smaller in the comparison to the mucosa of the proximal small intestine[146]. Further experiments are needed to fully understand SECO absorption and disposition characteristics following oral administration.

A non-compartmental approach was used to determine SECO PK parameters. Systemic exposure to SECO after oral bolus is much lower compared to intravenous administration

resulting in low oral bioavailability. SECO exhibits an extensive volume of distribution consistent with earlier studies on lignan disposition in the rat[107,109]. A relatively short half-life is similar to the half-lives of other plant lignans, such as pinoresinol, lariciresinol and 7-hydroxymatairesinol in humans[121]. Secondary serum concentration peaks indicate the presence of enterohepatic recirculation also observed by Penalvo *et al.*[121] for SECO and by Kuijsten *et al.*[113] for ED and EL in humans.

5.3.2 The 48-hour Pharmacokinetic Study

An additional 48-hour study was conducted in order to monitor the ED and EL concentration profile. Unfortunately, I was not able to quantify enterolignans in the majority of the samples. ED was detected in samples starting from 8 hours after the dose administration and was quantified at 21-24 hour time points following IV bolus only. EL was detected in samples from 15 to 36 hours post dose. Unfortunately, the developed method has lower sensitivity for EL, which could explain why EL was not quantified. ED and EL were found to undergo extensive glucuronidation in human and rhesus monkey hepatocytes[97]. Study on enterolignan intestinal metabolism using human Caco-2 cells also showed that EL is more readily conjugated than ED, which might contribute to my inability to quantify EL in the serum samples[98]. The delayed appearance of ED and EL is consistent with the results of PK studies in humans[113,120,121]. I also attempted to evaluate conjugated lignans by incubating serum with β-glucuronidase before analysis; however, interfering endogenous peaks that may have arisen from rat diet precluded quantification of the conjugated lignans.

Interestingly, unconjugated SECO was found in the systemic circulation up to 21 hours after the dose, with one animal showing significant quantities 36 hours post dose. This finding gives a new prospective on a possible biologically active lignan molecule. Felmlee *et al.*[1] observed that oral administration of equimolar quantities of SDG or SECO to Wistar rats fed 1% cholesterol diet caused dose-dependent reductions in serum total and LDL-cholesterol levels, hepatic lipid accumulation and the rate of body-weight gain. Although the exposure to enterolignan conjugates is reported to be much higher than to plant lignan conjugates across species, glucuronide formation usually serves as an elimination pathway for xenobiotics[94,121,156]. Glucuronides are polar, water-soluble, usually pharmacologically inactive compounds that are eliminated from the body more easily than their aglycones[156]. To my knowledge, no studies have assessed the concentration profile of unconjugated ED and EL, while my experiments indicate

that exposure to unconjugated SECO in rats is much higher than to unconjugated ED or EL. Moreover, to date no information is available on the pharmacological activity of lignan glucuronides as well. Therefore, the hypocholesterolemic effect in rats observed by Felmlee *et al.*[1] could be attributed at least partially to SECO. Recent study on chronic exposure to rye lignans in pigs also indicated high levels of plant lignans in systemic circulation and especially in bile (up to 77%)[94], warranting further experiments on lignan metabolism.

6. CONCLUSIONS AND FUTURE DIRECTIONS

The purpose of this research was to determine the pharmacokinetic parameters of SECO, the aglycone of the flaxseed lignan, SDG, in the rat animal model to help better understand lignan pharmacology. To complete the project, I needed sufficient quantity of pure SECO for the pharmacokinetic study, which is not readily available on the market. I also needed a simple and reliable method for lignan quantification in rat serum.

For my first objective, pure SECO (≥95% purity) was produced by acid hydrolysis of purified SDG isolated from the starting lignan material (40% SDG) by preparative HPLC. The molecule identity was confirmed by quadrupole time-of-flight mass spectrometry in negative ionization mode. SECO purity was confirmed by UPLC analysis.

For my second objective, an HPLC method with fluorescence detection for SECO, ED and EL quantification in rat serum was developed and validated. The method is rapid, provides good analytical sensitivity, and requires small serum volumes compared to currently available HPLC methods for lignan analysis. Although methods for lignan quantification involving mass spectrometry offer higher sensitivity, they are not available to many laboratories and often require radiolabeled or deuterated standards that need to be additionally synthetized. The developed method also allows for the simultaneous determination of all main metabolites of SDG, the major lignan in flax, making it suitable for complete pharmacokinetic lignan analysis in rat serum.

For my third objective and overall purpose of the project, two PK studies were conducted in male Wistar rats following a single intravenous and oral bolus administration of SECO. Lignan pharmacokinetic parameters have been assessed based on the results of a 12-hour study. SECO exhibits two compartment model characteristics with a large volume of distribution and low oral bioavailability. Oral data suggest that SECO undergoes enterohepatic recirculation. The results of a 48-hour study indicate that SECO is present in the systemic circulation at least for 21 hours after the dose administration. ED was detected and quantified in the samples taken between 8 to 24 hours, and EL was detected in samples taken between 15 to 36 hours after the SECO administration. Study results suggest SECO might significantly contribute to the cholesterol lowering effect observed in rats fed cholesterol diet following once a day oral administration of SDG or SECO.

Additional pharmacokinetic studies with SDG, ED and EL are necessary to understand which lignan form may mediate the beneficial health effects of flaxseed. Complete analysis of

bile, urine and faeces, as well as a thorough investigation of enterohepatic circulation of lignans could contribute to the understanding of lignan efficacy and mechanism of action.

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