A Thesis

Entitled

A Systematic Investigation of the Sulfation of Esculetin by the Human Cytosolic Sulfotransferases (SULTs)

by

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Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Master of Science Degree in Pharmaceutical Sciences (Pharmacology/Toxicology)

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May 2017
An Abstract of

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Esculetin is a coumarin derivative present in several plants that have been used as herbal medicine in China for many years. Esculetin has been shown to have a wide spectrum of biologic activities. However, the sulfation of esculetin has not yet been investigated. The present study was designed to identify and characterize the human cytosolic sulfotransferases (SULTs) that have the capacity to sulfate esculetin. A systematic investigation revealed that of the thirteen known human SULTs, SULT1A1, SULT1A3 and SULT1C4 showed the strongest sulfating activity toward esculetin. The pH-dependence and the kinetic parameters of these three SULTs in mediating the sulfation of esculetin were analyzed. HepG2 human hepatoma cells and Caco-2 human intestinal epithelial cells were shown to be able to sulfate esculetin under metabolic conditions. In addition, of the four human organ specimens (intestine, kidney, liver, and lung cytosols) examined, liver and intestine cytosols showed considerably higher esculetin-sulfating activity than lung and kidney cytosols. Taken together, these results
providied valuable biochemical information for further investigation on the pharmacokinetics of esculetin *in vivo*. 
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List of Abbreviations

ADP ......................... Adenosine-5’-Diphosphate
APS ......................... Adenosine-5’-Phosphosulfate
ATP ......................... Adenosine-5’-Triphosphate

CaCl$_2$ ..................... Calcium chloride
cDNA ...................... Complementary Deoxyribonucleic Acid
CAPS ....................... 3-(cyclohexylamino)-1-propanesulfonic acid
CHES ...................... Sodium acetate, 2- (Cyclohexylamino) Ethanesulfonic Acid
CYP ....................... Cytochrome P-450

DMSO ...................... Dimethyl Sulfoxide
DNA ...................... Deoxyribonucleic Acid
DTT ....................... Dithiothreitol

E. coli ....................... Escherichia Coli

FBS ....................... Fetal bovine serum

HEPES ..................... N-2-Hydroxylpiperazine-N2-Ethanesulfonic

IPTG ...................... Isopropyl-d-thiogalactopyranoside

LB ......................... Lysogeny Broth
LD$_{50}$ .................... Lethal dose of substrate that kills 50% of test samples

MBP ....................... Maltose-Binding Protein
MEM ....................... Minimum essential medium
mEH ....................... Microsomal epoxide hydrolase.
MES ....................... 2- Morpholinoethanesulfonic Acid
MOPS ...................... 3-(N-Morpholino) Propanesulfonic Acid

NaCl ....................... Sodium chloride
NADPH ...................... Reduced nicotinamide adenine dinucleotide phosphate
NaF ....................... Sodium fluoride
OD$_{600}$ nm ................Optical Density at 600 nm wavelength

PAPS ..................3'-phosphoadenosine-5'-phosphosulfate
PCR ..................Polymerase Chain Reaction
PPI ..................Pyrophosphate

S.D ..................Standard Deviation
SDS ..................Sodium Dodecyl Sulfate
SDS–PAGE ..........Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
sEH .................Soluble epoxide hydrolase
SULTs ..............Human Cytosolic Sulfotransferases Enzymes
SULT1A1 ...........Human Cytosolic Sulfotransferases Family 1A Member 1
SULT1A2 ...........Human Cytosolic Sulfotransferases Family 1A Member 2
SULT1A3 ...........Human Cytosolic Sulfotransferases Family 1A Member 3
SULT1B1 ...........Human Cytosolic Sulfotransferases Family 1B Member 1
SULT1C2 ...........Human Cytosolic Sulfotransferases Family 1C Member 2
SULT1C3 ...........Human Cytosolic Sulfotransferases Family 1C Member 3
SULT1C4 ...........Human Cytosolic Sulfotransferases Family 1C Member 4
SULT1E1 ...........Human Cytosolic Sulfotransferases Family 1E Member 1
SULT2A1 ...........Human Cytosolic Sulfotransferases Family 2A Member 1
SULT2B1a ..........Human Cytosolic Sulfotransferases Family 2B Member 1a
SULT2B1b ..........Human Cytosolic Sulfotransferases Family 2B Member 1b
SULT4A1 ...........Human Cytosolic Sulfotransferases Family 4A Member 1
SULT6B1 ...........Human Cytosolic Sulfotransferases Family 6B Member 1

TAPS ..................3-[N-Tris-(hydroxymethyl) Methylamino]-propanesulfonic acid
TLC ..................Cellulose Thin-Layer Chromatography
Tris-HCl ............Trisaminomethane Hydrochloride

UDP ..................Uridine diphosphate
### List of Symbols

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<td>Liter</td>
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<tr>
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</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>SO$_3^-$</td>
<td>Sulfonate Group</td>
</tr>
<tr>
<td>SO$_4^{2-}$</td>
<td>Inorganic Sulfate</td>
</tr>
<tr>
<td>nmol</td>
<td>Nanomole</td>
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<tr>
<td>mmol</td>
<td>Millimole</td>
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</tr>
<tr>
<td>$V_{max}$</td>
<td>Maximum Velocity</td>
</tr>
<tr>
<td>$K_m$</td>
<td>Michaelis Constant</td>
</tr>
<tr>
<td>$V_{max}/K_m$</td>
<td>Catalytic Efficiency</td>
</tr>
<tr>
<td>α</td>
<td>Angle of incidence</td>
</tr>
<tr>
<td>β</td>
<td>Angle of distortion</td>
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</table>

xv
Chapter 1

1. Introduction

1.1 Metabolism of xenobiotics

The human body is exposed daily to numerous xenobiotics through different exposure routes. Metabolism of xenobiotics by various Phase I and Phase II xenobiotic-metabolizing enzymes (XMEs) is critical to the prevention of the accumulation of these substances to toxic levels. In doing so, XMEs actively alter the physical and chemical properties of xenobiotics by increasing their hydrophilicity to facilitate their excretion. In some cases, however, XMEs may render some substances more toxic. While XMEs are expressed in every tissue and organ in the body, the liver and gastrointestinal tract are considered the main sites for XMEs since the levels of these enzymes are the highest in these organs.

Based on the reactions they mediate, XMEs are divided into two groups, designated Phase I and Phase II enzymes (cf. Table 1.1). Reactions mediated by the Phase I enzymes include oxidation, reduction, and hydrolysis. Phase I enzymes generally lead to added functional groups such as –OH, –COOH, –SH, –O–, or –NH2. In Phase II reactions, additional groups are added to the Phase I reaction metabolite, thereby rendering them more hydrophilic and easily excreted.
Table 1.1: Xenobiotic-metabolizing enzymes (Goodman, et al., 2011).

<table>
<thead>
<tr>
<th>ENZYMES</th>
<th>REACTIONS</th>
</tr>
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<tbody>
<tr>
<td><strong>Phase I &quot;oxygenases&quot;</strong></td>
<td></td>
</tr>
<tr>
<td>Cytochrome P450s (P450 or CYP)</td>
<td>C and O oxidation, dealkylation, others</td>
</tr>
<tr>
<td>Flavin-containing monooxygenases (FMO)</td>
<td>N, S, and P oxidation</td>
</tr>
<tr>
<td>Epoxide hydrolases (mEH, sEH)</td>
<td>Hydrolysis of epoxides</td>
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<tr>
<td><strong>Phase II &quot;transferases&quot;</strong></td>
<td></td>
</tr>
<tr>
<td>Sulfotransferases (SULT)</td>
<td>Addition of sulfate</td>
</tr>
<tr>
<td>UDP-glucuronosyltransferases (UGT)</td>
<td>Addition of glucuronic acid</td>
</tr>
<tr>
<td>Glutathione-S-transferases (GST)</td>
<td>Addition of glutathione</td>
</tr>
<tr>
<td>N-acetyltransferases (NAT)</td>
<td>Addition of acetyl group</td>
</tr>
<tr>
<td>Methyltransferases (MT)</td>
<td>Addition of methyl group</td>
</tr>
<tr>
<td><strong>Other enzymes</strong></td>
<td></td>
</tr>
<tr>
<td>Alcohol dehydrogenases</td>
<td>Reduction of alcohols</td>
</tr>
<tr>
<td>Aldehyde dehydrogenases</td>
<td>Reduction of aldehydes</td>
</tr>
<tr>
<td>NADPH-quinone oxidoreductase (NQO)</td>
<td>Reduction of quinones</td>
</tr>
</tbody>
</table>

1.1.1 Phase I XMEs

Phase I XMEs consist of three superfamilies: cytochrome P-450s (CYPs), flavin-containing monooxygenases (FMOs), and epoxide hydrolases (EHs). The CYPs are considered the most common Phase I enzymes, being involved in the metabolism of the majority of xenobiotic compounds. Additionally, CYPs are known to be involved in the biosynthesis of certain endogenous compounds (Goodman, et al. 2011). Although CYPs are present in many organs in the body, the liver is the main location of CYPs and FMOs (Krishna and Klotz, 1994). FMOs are also important xenobiotic-metabolizing enzymes. The deficiency of FMO3 has been reported to cause fish-odor syndrome. The third superfamily of Phase I XMEs, epoxide hydrolases (EHs), are further divided into two
sub-groups: the soluble epoxide hydrolases (sEHs) and the microsomal epoxide hydrolases (mEHs), which play an important role in deactivating toxic metabolites produced by CYPs (Goodman, et al., 2011).

1.1.2 Phase II XMEs

Phase II enzymes are generally called the transferases since they mediate the transfer of functional groups from donor molecules to the Phase I reaction metabolites. In doing so, the transferases assist in increasing their hydrophilicity and excretion from the body. In this regard, transferases are considered to play a complementary role to that of Phase I enzymes. Phase II XMEs include the following superfamilies enzymes: the cytosolic sulfotransferases (SULTs), UDP-glucuronosyltransferases (UGTs), glutathione-S-transferases (GSTs), N-acetyltransferases (NATs), and methyltransferases (MTs). SULTs transfer the sulfonate group from 3’- phosphoadenosine 5’-phosphosulfate (PAPS), a cofactor, to the hydroxyl or amino group of aromatic and aliphatic compounds (cf. Figure 1-1). UGTs transfer glucuronic acid from UDP-glucuronic acid, a cofactor, to substrate compounds to produce glucuronides. GSTs transfer glutathione, which is synthesized from γ-glutamic acid, cysteine, and glycine, to reactive electrophiles. NATs transfer the acetyl group from acetyl-coenzyme A, a cofactor, to aromatic amino or hydrazine group of substrate compounds. MTs catalyze the transfer of a methyl group to produce methylated products. The rates of Phase II reactions are generally faster than the rates of Phase I reactions, which is believed to ensure the efficient elimination and detoxification of xenobiotics (Goodman, et al., 2011).
1.2 Physiological roles of sulfation

Biological sulfation was discovered in 1876 when phenol sulfate was isolated from the urine sample of a patient treated with phenol as an antiseptic (Baumann, 1876). Based on subcellular localization of sulfotransferases, they are divided in two major families: membrane-bound sulfotransferases and cytosolic sulfotransferases (SULTs). The membrane-bound sulfotransferases, which are localized in the Golgi apparatus of cells, are responsible for the sulfation of macromolecules such as glycosaminoglycans, glycoproteins, and tyrosines in proteins and peptides. Most sulfated products of the membrane-bound sulfotransferases are secreted by the Golgi apparatus and affect the biological activities of glycosaminoglycans, glycoproteins, and proteins. SULTs are responsible for the sulfation of low-molecular weight endogenous and exogenous compounds such as hormones and drugs (Strott, 20002; Falany, 2005; Falany, 1997).

Physiologically speaking, sulfation can deactivate or bioactivate different xenobiotics, and inactivate endogenous hormones and catecholamines (Klaassen and Boles, 1997). Some compounds, such as N-hydroxy-2-acetylaminofluorene can be
bioactivated via sulfation reaction, catalyzed by certain SULTs, to generate reactive electrophilic, mutagenic, and carcinogenic sulfuric acid ester metabolites, which may react with cellular nucleophiles to produce mutagenic and/or cytotoxic responses (Miller, 1994).

Based on the active groups present in the subjected agents of the SULTs, sulfation reactions of xenobiotics are classified into three primary categories and include: \( O \)-sulfation (ester), \( N \)-sulfation (amide), and \( S \)-sulfation (thioester) (Strott, 2002).

As mentioned above, SULTs need PAPS, the universal sulfate donor, to catalyze the sulfation reaction. The synthetic pathway and characteristics of PAPS were successfully identified in the 1950s (Robbins and Lipmann 1956; Baddiley, et al., 1958; Baddiley, et al., 1959). In detail, PAPS is synthesized in the cytosol and two enzymes are involved in its biosynthesis (cf. Figure 1-2). First, adenosine-5’-triphosphate (ATP) sulfurylase mediates the generation of adenosine-5’-phosphosulfate (APS) and inorganic phosphate, pyrophosphate (PPi), by catalyzing the reaction of inorganic sulfate (\( SO_4^{2-} \)) with ATP. Second, APS kinase catalyzes the reaction of APS with another molecule of ATP to form PAPS and adenosine-5’-diphosphate (ADP). Inorganic sulfate (\( SO_4^{2-} \)), which is available from the diet and catabolism of proteins and sugar sulfates, is required to complete synthesis of PAPS in the cytosol (Strott, 2002; Klaassen and Boles, 1997). The role of SULTs in the sulfation reactions is to catalyze the conjugation of a sulfonate group (\( SO_3^- \)), that is received from PAPS, to a nucleophilic moiety of subjected compounds (cf. Figure 1-1; Chapman, et al., 2004).


1.2.1 Cytosolic sulfotransferases (SULTs)

SULTs are involved in detoxification, hormone regulation, and drug metabolism by catalyzing the physiological sulfation processes (Chapman et al., 2004). Some recent studies revealed that sulfation may play a major role in the homeostasis of hormones and other endogenous compounds as well as in detoxification during fetal development. So, SULTs are considered a major detoxification enzyme system during the developmental process since they are highly expressed in the fetus. In contrast, the UDP-glucuronosyltransferases, which are not expressed significantly until the neonatal period (Richard, et al., 2001; Coughtrie, 2002).

In structure, all SULTs are nearly spherical and contain a single α/β fold with a central four or five stranded parallel β-sheet surrounded by α helix. Besides, the PAPS-binding region and its structure are conserved at the amino acid sequence level for all SULTs. The substrate-binding region, however, is considered where SULTs differ in their structures (Chapman et al., 2004).
Thirteen human SULTs have been discovered and divided into four major families, designated SULT1, SULT2, SULT4, and SULT6, based on their amino acid sequences and structure analysis. The biological activities of members of different SULT families varies in accordance to the amino acid sequence (Coughtrie 2002; Allali-Hassani, et al., 2007; Strott, 2002; Falany, 2005).

1.2.1.1 SULT1 family

The SULT1 family includes eight members that are further classified into four subfamilies: SULT1A1, SULT1A2, and SULT1A3; SULT1B1; SULT1C1, SULT1C2, and SULT1C3; and SULT1E1 (Allali-Hassani, et al., 2007). SULT1As are responsible for sulfating a wide variety of small phenolic compounds and conjugating amines to produce sulfamates. The gene sequence of SULT1A1, SULT1A2, and SULT1A3 are more than 92% identical. SULT1A1 and SULT1A3 are expressed in human platelets, the liver, GI tract, and brain. The expression of SULT1A2 in human tissues is low compared to that of SULT1A1 and SULT1A3 (Gamage, et al., 2006; Strott, 2002; Falany, 2005). SULT1B1 has the capacity to sulfate thyroid hormones and DOPA. The amino acid sequence of SULT1B1 is 52-53% similar to SULT1A1, SULT1A2, and SULT1A3. Its highest expression is observed in the colon, small intestine, spleen, and leukocytes. It is also detected in the liver (Gamage, et al., 2006; Strott, 2002; Glatt, at al., 2001; Falany, 2005). SULT1Cs have the capacity to activate potent procarcinogens such as N-hydroxy-2-acetylnaminofluorene at high concentration, and catalyze sulfation of some xenobiotics. The amino acid sequence of SULT1C1 and SULT1C2 are 63% identical. SULT1Cs are highly expressed in the liver, thyroid, kidney, heart, ovarian tissue, and fetal liver, spleen, and kidney (Runge-Morrsi and Kocarek, 2013; Pai, et al., 2001; Falany, 2005). SULT1E
is considered a specialized SULT that sulfates mostly estrogenic steroids. The SULT1E1 amino acid sequence is 44-56% identical to that of the other members of the SULT1 family. It is expressed in the liver, endometrium, jejunum, and testis, and breast (Glatt, et al., 2001; Strott, 2002; Falany, 2005).

1.2.1.2 SULT2 family

The SULT2 family includes three members that are classified into two subfamilies SULT2A1 and SULT2B. The SULT2 family members are responsible for conjugating the sulfonate group to hydroxyl groups of steroids and sterols, such as androsterone, allopregnanolone, and dehydroepiandrosterone (DHEA) (Allali-Hassani, et al., 2007; Strott, 2011). SULT2A1 is able to sulfate bile acid in human liver. SULT2B1b has the capacity to sulfate cholesterol. SULT2A1 is expressed in the liver, adrenal cortex, and intestine, whereas SULT2Bs are expressed in the placenta, prostate, and trachea (Glatt, Boeing et al., 2001, Falany, 2005). The SULT2A1 amino acid sequence is 48% identical to SULT2B1s (Falany, 2005).

1.2.1.3 SULT4 family

SULT4 family includes only one member, designated SULT4A1, which is highly conserved among all vertebrates and binds well to the neurotransmitters epinephrine and norepinephrine. Since it is only expressed in the brain, it likely has a critical biological function therein. However, its physiological properties have not been completely identified (Allali-Hassani, et al., 2007).

1.2.1.4 SULT6 family
SULT6 family also includes only one member, designated SULT6B1, which is specifically expressed in kidney and testis (Takahashi, et al., 2009). The protein and enzymatic activity of SULT6 has not yet been identified (Allali-Hassani, et al., 2007).

Figure 1-3: Chemical Structure of esculetin (Lin, et al., 2000).

1.3 Esculetin and its biological activities

Esculetin (6,7-dihydroxy coumarin; cf. Figure 1-3) is a coumarin derivative and has been categorized under the group of simple coumarins, which are also known as cichorigenin (Kadakol, et al., 2016). Esculetin has been found in several plants such as *Cortex fraxini* (Zhang, et al., 2000; Wu, et al., 2007), *Viola yedoensis* (Oshima, et al., 2013), *Aesculus hippocastanum* (Wilkinson and Brown, 1999), *Artemisia capillaries*, *Artemisia scoparia*, *Ceratostigma willmottianum*, the leaves of *Citrus limonia*, and *Cichorium intybus* (Kadakol, et al., 2016). Esculetin has been reported in many studies to possess a variety of biological effects including antioxidant, (Mao, et al., 2015; Kim, et al., 2008) anti-inflammatory (Witaicenis, et al., 2010; Mao, et al., 2015), chemoprotective (Matsunaga, et al., 1998), anti-tumor (Mao, et al., 2015), antiproliferative (Dai Rong, et al., 2009) antibacterial (Duncan, et al., 2004), neuroprotective (Wang, et al., 2012), anti-anxiety (Sulakhiya, et al., 2016), anti-depressant (Martin-Aragon, et al., 2016), anti-Alzheimer’s disease (Ali, et al., 2016), antihyperglycemic (Prabakaran and Ashokkumar,
Antioxidant activity of esculetin is attributed to its ability to inhibit several pathways which participate in oxidative stress conditions such as B-cell lymphoma 2/Bcl2-like protein (Bcl-2/ Bax) pathway, epidermal growth factor receptor/phosphatidylinositol/Akt (EGFR/ PI3 K/Akt) pathway, peroxisome proliferator-activated receptor gamma/transforming growth factor beta (PPAR-γ/TGF-β), and mitogen activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway (cf. Figure 1-4; Kadakol, et al., 2016).

1.3.1 Metabolic pathways of esculetin

Since esculetin is a coumarin-related compound, the main metabolic pathways of coumarin in Phase I reaction is hydroxylation as catalyzed by hepatic CYP2A6 to yield 7-hydroxycoumarin or 3-hydroxycoumarin (cf. Figure 1-5) and then further conjugated with D-glucuronic acid and/or sulfate at 7-OH (Egan, et al., 1990; Miles, et al., 1990;
Zhu, et al., 2015). In addition, esculetin can be metabolized to scopoletin in vivo via methylation. The metabolite scopoletin, which has been shown to exhibit antioxidant, anticancer, and anti-inflammatory effects, might play a synergistic effect with esculetin in vivo (Wang, 2016). Moreover, UGT1A6 and UGT1A9 have been shown to display the strongest activity to mediate 7-O-monoglucuronidation of esculetin in vitro (cf. Figure 1-6; Zhu, et al., 2015).

Nevertheless, systemic studies on the metabolites of esculetin in vivo are still necessary to clarify further the metabolism of esculetin and its metabolites in order to evaluate their efficacy and safety.

Figure 1-5: Formation of 7 and 3-hydroxycoumarin occurs in Phase I reaction (Born, Hu, et al. 2000).

Figure 1-6: Proposed metabolic pathways of esculetin (Wang, 2016).
1.3.2 Kinetic parameters of esculetin in animals

In previous studies, pharmacokinetic parameters of orally administered esculetin were investigated in plasma and tissues of rats. The maximum plasma concentration ($C_{\text{max}}$) value was 173.3 ng/mL, and elimination half-life ($t_{1/2}$) was 45 min. The area under the plasma concentration–time curve (AUC) value was 5167.5 ng · min/mL (Kim, et al., 2014). Accordingly, the pharmacokinetic profile of esculetin implied that its absorption and elimination from plasma is very rapid, and it is quickly distributed and eliminated from tissue. The acute toxicity of esculetin has been investigated in mice after oral and intraperitoneal routes of administration. The oral route LD$_{50}$ was > 2000 mg/kg, and the intraperitoneal route LD$_{50}$ was 1450 mg/kg (Tubaro, et al., 1988).

13.3 The chemical synthesis of esculetin

In light of all of its benefits, a number of chemically synthesis procedures have been developed to produce esculetin. As such, there are three primary chemical processes used to produce esculetin, and those processes include the following procedures as depicted by the figures below:

1- The use of p-benzoquinone, acetic anhydride and sulfuric acid to synthesize 1, 2, 4-phloroglucinol triacetate intermediate, which was then mixed with concentrated sulfuric acid and malic acid to produce esculetin (Mao, et al., 2015).

Figure 1-7: Chemically synthesis of esculetin (Mao, et al., 2015).
2- The use of p-benzoquinone, acetoacetate, concentrated sulfuric acid to produce 1,2,4-phloroglucinol triacetate intermediate and 1,2,4-benzenetriol, which is then combined with sulfuric acid and malic acid to synthesize esculetin (Mao, et al., 2015).

![Chemical synthesis of esculetin by unifactor and multifactor orthogonal experiment](image1)

Figure 1-8: Chemically synthesis of esculetin by unifactor and multifactor orthogonal experiment (Mao, et al., 2015).

3- The use of 1,2,4-benzenetriol and ethyl propionate, thereafter using ZnCl$_2$ as a catalyst under 400W microwave irradiation to produce esculetin (Mao, et al., 2015).

![Chemically synthesis of esculetin under microwave irradiation and used ZnCl2 as catalyst](image2)

Figure 1-9: Chemically synthesis of esculetin under microwave irradiation and used ZnCl2 as catalyst (Mao, et al., 2015).
Objectives and Goals

As mentioned above, the main Phase I metabolic pathway for coumarin is hydroxylation as catalyzed by hepatic cytochrome P450 (CYP) 2A6, which yields 7-hydroxycoumarin or 3-hydroxycoumarin. These hydroxylated coumarin derivatives may then be further conjugated with D-glucuronic acid and/or sulfate at 7-OH. UGT1A6 and UGT1A9 have been reported to be able to mediate 7-O-monoglucuronidation of esculetin. However, the human enzymes, involved in the sulfation of esculetin, have not been identified. Accordingly, the aim of this thesis is to identify and characterize the human cytosolic sulfotransferases (SULTs) that have the capacity to sulfate esculetin in the presence of PAPS as a cofactor in vitro.
Chapter 2

2. Materials and Methods

2.1 Materials

Esculetin was purchased from Enzo Life Science Inc. (Farmingdale, NY). Adenosine-5’-triphosphate (ATP), dithiothreitol (DTT), sodium acetate, dimethyl sulfoxide (DMSO), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), 2-(cyclohexyl-amino) ethanesulfonic acid (CHES), β-naphthol, 3-(N-morpholino)propanesulphonic acid (MOPS), N-2-hydroxyethylpiperazine-N2-ethanesulfonic acid (HEPES), 2-morpholinoethanesulfonic acid (MES), N-[Tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid (TAPS), isopropyl-β-thiogalactopyranoside (IPTG), Trizma base, minimum essential medium (MEM), fetal bovine serum (FBS), streptomycin sulfate, sodium fluoride (NaF), and penicillin G were purchased from Sigma Chemical Company (St. Louis, MO). Ecolume scintillation cocktail was purchased from MP Biomedicals (Solon, OH). Carrier-free sodium $^{35}$S sulfate was a product of American Radiolabeled Chemicals (St. Louis, MO). Recombinant human bifunctional ATP sulfurylase/adenosine 5’-phosphosulfate kinase, which was used to synthesize PAP$^{[35]S}$, was prepared as previously described (Yanagisawa, et al., 1998). Cellulose thin-layer
chromatography (TLC) plates were products of EMD Chemicals (Gibbstown, NJ). TRI reagent was obtained from Molecular Research Center, Inc. (Cincinnati, OH). Taq DNA polymerase was from Promega Corporation (Madison, WI). Oligonucleotide primers were synthesized by MWG Biotech (High Point, NC). Recombinant human SULTs were cloned, expressed, and purified as described previously (Suiko, et al., 2000; Pai, et al., 2002; Sakakibara, et al., 1998; Kurogi, et al., 2012). Caco-2 human intestinal epithelial cells (ATCC-HTB-37) and HepG2 human hepatoma cell line (ATCC HB-8065) were from American Type Culture Collection (Manassas, VA). Kidney S9 fraction (Lot No. 0510093), pooled human lung S9 fraction (Lot No. 0710281), small intestine (duodenum and jejunum) S9 fraction (Lot No. 0710351), and liver cytosol (Lot No. 09103970) were from XenoTech, LLC (Lenexa- KS). All other chemicals used were of the highest grade commercially available.

2.2 Methods

2.2.1 Molecular cloning of cDNAs encoding human SULTs

Sets of sense and antisense oligonucleotide primers, based on 5′- and 3′- coding regions of reported nucleotide sequences of different human SULTs, were synthesized with BamHI and EcoRI restriction sites incorporated at the ends. With respective sets of oligonucleotides as primers, PCRs in 10 µl reaction mixtures were carried out under the action of LA Taq DNA polymerase using individual human organ first-strand cDNAs as templates. Amplification conditions were 27 cycles of 1 min at 94°C for denaturation, 1 min at 56°C for annealing, and 2 min at 72°C for extension. The final reaction mixtures were applied onto a 1.2 % agarose gel and separated by electrophoresis. The discrete
PCR product bands, visualized by ethidium bromide staining, were excised from the gel and the DNA fragments therein were isolated by spin filtration. After EcoRI or BamHI digestion, the PCR products were subcloned into the EcoRI or BamHI site of pGEX-2TK. To verify their authenticity, the cDNA inserts were subjected to nucleotide sequencing.

### 2.2.2 Expression and purification of the recombinant human SULTs

Competent *E. coli* BL21 cells were transformed with pGEX-2TK harboring full-length cDNA encoding individual human SULTs. Transformed cells, grown in 1 liter LB medium supplemented with 100 mg/ml ampicillin and after the cell density reached 0.6 OD$_{600}$ nm, 0.1 mM IPTG was added to induce the production of recombinant protein. After a 5-hour induction at 37°C, the cells were collected by centrifugation and homogenized in 20 ml ice-cold lysis buffer using an Aminco French press. 20 µl of a protease inhibitor mixture (Roche Diagnostics) were added to the crude homogenate thus prepared was subjected to centrifugation at 10,000g for 30 min at 4°C. The supernatant collected was fractionated using 0.5 ml of glutathione–Sepharose, and the bound glutathione S-transferase fusion protein was treated with 2 ml of a thrombin digestion buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 2.5 mM CaCl2) containing 5 unit/ml bovine thrombin. Following a 30-min incubation at room temperature with constant agitation, recombinant SULT enzyme present in the supernatant was collected and analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and examined for enzymatic activities toward esculetin (Suiko, et al., 2000; Pai, et al., 2002; Sakakibara, et al, 1998; Kurogi, et al., 2012).
2.2.3 Sulfotransferase assay

Radioactive PAP\[^{35}\text{S}\] was used as the sulfate donor to assay for esculetin-sulfating activity of purified recombinant human SULTs. Esculetin was dissolved in DMSO, and diluted to a final concentration 50 µM in the final reaction mixture. A control with DMSO replacing the substrate was also prepared. Purified SULT1A1, SULT1A2, SULT1A3, SULT1B1, SULT1C2, SULT1C3, SULT1C4, SULT1E, SULT2A1, SULT2B1a, SULT2B1b, SULT4A1, and SULT6B1 were tested in the assay. The standard reaction mixture for the enzymatic assay, prepared in a final volume of 20 µl, contained 50 mM HEPES at pH 7.0, 14 µM of PAP\[^{35}\text{S}\] (15 Ci/mmol), 1 mM DTT and 50 µM of esculetin. The reaction was started by the addition of 2 µl of the SULT enzyme, allowed to proceed at 37°C for 10 minutes and terminated by heating the tube containing the reaction mixture on a heating block at 100°C for 3 minutes. Precipitates, formed in the heated reaction mixture, were cleared by centrifugation at 13,000×g for 3 minutes. Subsequently, 1 µl of the supernatant reaction mixture was spotted on a cellulose TLC plate, and the plate was subjected to TLC analysis using a solvent system containing n-butanol / isopropanol / 88% formic acid / Milli-Q water in a ratio of (3:1:1:1; by volume). Afterwards, the TLC plate was air-dried and autoradiographed by using an X-ray film. The autoradiograph taken from the TLC plate was used to locate the radioactive spot corresponding to the sulfated product, and the located spot was cut from the TLC plate, eluted in 0.5 ml Milli-Q water in a glass vial, and mixed thoroughly with 2 ml of Ecolume scintillation liquid. The radioactivity was counted by using a liquid scintillation counter. The results obtained were used to calculate the specific activity in the unit of sulfated product formed/minute/mg of SULTs.
2.2.4 pH-Dependence Study

pH-dependence of the esculetin-sulfating activity of human SULT1A1, SULT1A3, and SULT1C4 was examined by using different buffers (sodium acetate at 4.5, 5, 5.5; MES at 5.5, 6, 6.5; MOPS at 6.5, 7, 7.5; HEPES at 7, 7.5, 8; TAPS at 8, 8.5, 9; CHES at 9, 9.5, 10; CAPS at 10, 10.5, 11, 11.5), instead of 50 mM HEPES pH 7.0, in assay mixtures. The experimental procedure for pH-dependence studies was the same as described above, except for the buffer used.

2.2.5 Kinetic Studies

To study the kinetic parameters of human SULT1A1, SULT1A3, and SULT1C4 in mediating the sulfation of esculetin, varying concentrations of esculetin and 50 mM HEPES buffer at pH 7.4 were used. The reaction conditions were the same as those previously described in the sulfotransferase assay. The results obtained were analyzed based on Michaelis-Menten equation with nonlinear-regression using GraphPad Prism 5 software program.

2.2.6 Sulfation of esculetin by human organ samples

Cytosol prepared from human lung, liver, kidney, and intestine, instead of purified human SULTs, were tested based on above-mentioned assay procedure, with 50 mM HEPES buffer at pH 7.4 as the buffer.
2.2.7 Metabolic labelling of HepG2 human hepatoma cells and Caco-2 human intestinal epithelial cells

HepG2 cells and Caco-2 cells were maintained, under a 5% CO$_2$ atmosphere at 37°C, in MEM supplemented with 10% FBS, penicillin G (30 µg/ml) and streptomycin sulfate (50 µg/ml). Confluent cells grown in individual wells of a 24-well culture plate, pre-incubated in sulfate-free (prepared by omitting streptomycin sulfate and replacing magnesium sulfate with magnesium chloride) MEM without FBS for 4 h, were labeled with 0.25 ml aliquots of the same medium containing $[^{35}S]$sulfate (0.3 mCi/ml) plus different concentrations (0.5, 1.25, 2.5, and 5 µM) of esculetin. At the end of an 18-h labelling period, the media were collected, spin-filtered to remove high-molecular weight $[^{35}S]$sulfated macromolecules, and subjected to thin-layer analysis for $[^{35}S]$sulfated esculetin based on the procedure described above.

2.2.8 Miscellaneous methods

PAP$[^{35}S]$ was synthesized from ATP and carrier-free $[^{35}S]$sulfate using the recombinant human bifunctional ATP sulfurylase/adenosine 5’-phosphosulphate kinase and its purity determined as previously described (Yanagisawa, et al., 1998). The synthesized PAP$[^{35}S]$ was adjusted to the required concentration and a specific activity of 15 Ci/mmol at 1.4 mM by the addition of non-radioactive PAPS. Protein determination was based on the method of Bradford with bovine serum albumin as the standard (Bradford, 1976).
Chapter 3

3. Results and Discussion

3.1 Results

3.1.1 Survey of sulfating activity of the human SULTs toward esculetin

A systematic study of the esculetin-sulfating activity of all thirteen known human SULTs was performed, as described in chapter 2, in order to identify those that are capable of catalyzing the sulfation of esculetin. With 50 µM of esculetin as substrate, results revealed that five, SULT1A1, SULT1A2, SULT1A3, SULT1B1, and SULT1C4, of the thirteen human SULTs exhibited sulfating activities toward esculetin. Of these five SULTs, SULT1A1, SULT1A3, and SULT1C4 displayed considerably stronger esculetin-sulfating activity than the other two SULTs. Activity data compiled in Table 3.1 showed that the esculetin-sulfating activity of SULT1A1, SULT1A3, and SULT1C4 were 32.43, 15.30, 9.77 nmol/min/mg at neutral pH, respectively.
Table 3.1: Specific Activity of Human SULTs with Esculetin as a Substrate.

<table>
<thead>
<tr>
<th>Esculetin concentration (µM)</th>
<th>SULT1A1</th>
<th>SULT1A2</th>
<th>SULT1A3</th>
<th>SULT1B1</th>
<th>SULT1C4</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>32.43±0.40</td>
<td>0.64±0.11</td>
<td>15.30±0.66</td>
<td>1.60±0.09</td>
<td>9.77±0.22</td>
</tr>
</tbody>
</table>

3.1.2 pH-dependence studies of human SULT1A1, SULT1A3, and SULT1C4

The pH-dependence of SULT1A1, SULT1A3 and SULT1C4 in mediating the sulfation of esculetin were analyzed to examine further the sulfating activity of esculetin by SULT1A1, SULT1A3, and SULT1C4. The pH-dependence studies were performed under the same standard assay conditions as described in Chapter 2. As shown in Figure 3-1, 3-2, and 3-3, variations in the pH-dependence of the sulfation of esculetin among these three human SULTs were found. The results from the pH-dependence experiments revealed that SULT1A1 showed a pH optimum spanning pH 5.5 to 6.5 (cf. Figure 3-1), whereas SULT1A3 exhibited a pH optimum at pH 6.5 (cf. Figure 3-2). SULT1C4 showed a pH optimum spanning pH 6.5 to 8 (cf. Figure 3-3).
Figure 3-1: pH-dependence of the sulfating activity of the human SULT1A1 with 50 μM of esculetin as a substrate. Enzymatic assays were carried out under the standard conditions as described in Chapter 2 using different buffer systems. Data shown represents calculated mean ± S.D derived from three independent experiments.
Figure 3-2: pH-dependence of the sulfating activity of the human SULT1A3 with 50 µM of esculetin as a substrate. Enzymatic assays were carried out under the standard conditions as described in Chapter 2 using different buffer systems. Data shown represents calculated mean ± S.D derived from three independent experiments.
Figure 3-3: pH-dependence of the sulfating activity of the human SULT1C4 with 50 µM of esculetin as a substrate. Enzymatic assays were carried out under the standard conditions as described in Chapter 2 using different buffer systems. Data shown represents calculated mean ± S.D derived from three independent experiments.
3.1.3 Kinetic studies of human SULT1A1, SULT1A3, and SULT1C4

The kinetic parameters of esculetin sulfation by the SULT1A1, SULT1A3, and SULT1C4 were analyzed to investigate the sulfation of esculetin in more detail. In these experiments, different concentrations of esculetin and 50 mM HEPES at pH 7.4 were used in the enzymatic assay procedure as described in Chapter 2. The final concentrations of esculetin, which were used in the experiments, are listed in Table 3.2. Data obtained from these experiments were processed using the GraphPad Prism 5 software program to generate the best fitting curves for the Michaelis-Menten equation with non-linear regression in order to calculate the values of $K_m$, $V_{max}$, and $V_{max}/K_m$ for the SULT1A1, SULT1A3, and SULT1C4 in mediating the sulfation of esculetin. Data on the sulfation of esculetin by each of the three human SULTs were fitted to hyperbolic kinetic curves (Michaelis-Menten kinetics), which was further confirmed by the Lineweaver-Burk double-reciprocal plots. Figure 3-4, 3-6, and 3-8 show the saturation kinetics of the SULT1A1, SULT1A3, and SULT1C4, respectively. Figure 3-5, 3-7, and 3-9 show the Lineweaver-Burk double-reciprocal plots, where $1/[S]$ was plotted against $1/v$, of the SULT1A1, SULT1A3, and SULT1C4, respectively. Calculated values of $K_m$, $V_{max}$, and $V_{max}/K_m$ for the SULT1A1, SULT1A3, and SULT1C4 are compiled in Table 3.3.
Table 3.2: List of the Concentrations of Esculetin used in the Kinetic Studies of the human SULT1A1, SULT1A3, and SULT1C4.

<table>
<thead>
<tr>
<th>SULTs</th>
<th>Final Concentrations of esculetin (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SULT1A1</td>
<td>0.625, 0.714, 0.83, 1, 1.25, 1.66, 2.5, 5, 10</td>
</tr>
<tr>
<td>SULT1A3</td>
<td>8.33, 9.09, 10.00, 11.1, 12.50, 14.29, 16.60, 20, 25, 33, 50, 100</td>
</tr>
<tr>
<td>SULT1C4</td>
<td>4.54, 5, 5.5, 6.25, 7.14, 8.33, 10.00, 12.50, 16.60, 25, 50</td>
</tr>
</tbody>
</table>

Figure 3-4: The figure shows the saturation curve analysis of the sulfation of esculetin by human SULT1A1. The fitting curve was generated based on Michaelis-Menten equation with non-linear regression using GraphPad Prism5 software. The velocity of the reaction is indicated as nmol/min/mg of the enzyme. Data shown represent calculated mean ± S.D derived from three experiments.
Figure 3-5: Lineweaver-Burk double-reciprocal plot of human SULT1A1 using esculetin as the substrate. The concentrations of esculetin tested were 0.625, 0.714, 0.83, 1, 1.25, 1.66, 2.5, 5, and 10 µM. The velocity of the reaction is expressed as nmol/min/mg of enzyme. Each data point shown represents mean ± S.D of the mean derived from three measurements.
Figure 3-6: The figure shows the saturation curve analysis of the sulfation of esculetin by human SULT1A3. The fitting curve was generated based on Michaelis-Menten equation with non-linear regression using GraphPad Prism5 software. The velocity of the reaction is indicated as nmol/min/mg of the enzyme. Data shown represent calculated mean ± S.D derived from three experiments.
Figure 3-7: Lineweaver-Burk double-reciprocal plot of human SULT1A3 using esculetin as the substrate. The concentrations of esculetin tested were 8.33, 9.09, 10.00, 11.1, 12.50, 14.29, 16.60, 20, 25, 33, 50, and 100 µM. The velocity of the reaction is expressed as nmol/min/mg of enzyme. Each data point shown represents mean ± S.D of the mean derived from three measurements.
Figure 3-8: The figure shows the saturation curve analysis of the sulfation of esculetin by human SULT1C4. The fitting curve was generated based on Michaelis-Menten equation with non-linear regression using GraphPad Prism5 software. The velocity of the reaction is indicated as nmol/min/mg of the enzyme. Data shown represent calculated mean ± S.D derived from three experiments.
Figure 3-9: Lineweaver-Burk double-reciprocal plot of human SULT1C4 using esculetin as the substrate. The concentrations of esculetin tested were 4.54, 5, 5.5, 6.25, 7.14, 8.33, 10.00, 12.50, 16.60, 25, and 50 µM. The velocity of the reaction is expressed as nmol/min/mg of enzyme. Each data point shown represents mean ± S.D of the mean derived from three measurements.

Table 3.3: Kinetic Parameters of the Sulfation of Esculetin by human SULT1A1, SULT1A3, and SULT1C4.

<table>
<thead>
<tr>
<th>SULTs</th>
<th>$V_{max}$ (nmol/min/mg)</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SULT1A1</td>
<td>32.83±2.14</td>
<td>2.229±0.33</td>
<td>14.72</td>
</tr>
<tr>
<td>SULT1A3</td>
<td>19.07±1.1</td>
<td>95.49±7.9</td>
<td>0.199</td>
</tr>
<tr>
<td>SULT1C4</td>
<td>53.93±1.6</td>
<td>34.29±1.6</td>
<td>1.57</td>
</tr>
</tbody>
</table>
3.1.4 Sulfation of esculetin by human organ samples

To investigate further whether sulfation of esculetin may occur in vivo, the enzymatic assay procedure described in Chapter 2 was performed using cytosol fractions prepared from human lung, liver, kidney, or intestine. These experiments were performed at 50 mM HEPES at pH 7.4, and the concentration of esculetin tested was 50 µM. The specific activities of intestine and liver cytosols were 552.98 and 261.00 pmol/min/mg protein, respectively. The specific activities of lung and kidney cytosols were 34.45 and 42.20 pmol/min/mg protein, respectively. Table 3.4 shows the activity data obtained from these experiments.

Table 3.4: Sulfating Activity of human Lung, Liver, Kidney and Intestine cytosols with Esculetin as a Substrate.

<table>
<thead>
<tr>
<th>Organs</th>
<th>Specific activity (pmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestine</td>
<td>552.98±4.47</td>
</tr>
<tr>
<td>Liver</td>
<td>261.00±4.52</td>
</tr>
<tr>
<td>Kidney</td>
<td>42.20±2.16</td>
</tr>
<tr>
<td>Lung</td>
<td>34.45±3.62</td>
</tr>
</tbody>
</table>

3.1.5 Metabolic labelling of HepG2 human hepatoma cells and Caco-2 human intestinal epithelial cells

Cultured HepG2 human hepatoma cells and Caco-2 human intestinal epithelial cells were used to evaluate whether esculetin sulfation may occur under metabolic conditions. HepG2 cells and Caco-2 cells were routinely maintained, under a 5% CO2
atmosphere at 37°C, in MEM supplemented with 10% FBS, penicillin G (30 µg/mL) and streptomycin sulfate (50 µg/mL). Confluent HepG2 cells and Caco-2 cells grown in individual wells of a 24-well culture plate, pre-incubated in sulfate-free MEM without FBS for 4 h, then were labeled with the same medium containing [³⁵S]sulfate (0.3 mCi/mL) plus different concentrations (0.5, 1.25, 2.5, and 5 µM) of esculetin. Following an 18-h incubation period, the spent labeling medium samples were collected and analyzed by TLC based on the procedure described in Chapter 2. As shown in Figure 3-10, [³⁵S]sulfated esculetin was detected in spent labeling medium containing as low as 0.5 µM of esculetin, which continued to increase with increasing concentrations of esculetin added to the labeling media.
Figure 3-10: Analysis of the $^{35}$S sulfated product generated and released by HepG2 human hepatoma cells and Caco-2 human intestinal epithelial cells labeled with $^{35}$S sulfate in the presence of esculetin. Confluent HepG2 cells and Caco-2 cells were labeled with $^{35}$S sulfate in the presence of different concentrations (0.5, 1.25, 2.5, and 5 $\mu$M) of esculetin. At the end of an 18-h labelling, spent labelling media were collected and analyzed by TLC. The figure shows the autoradiograph taken from the cellulose plate used for the TLC analysis. The solid arrows indicate the sulfated products of esculetin.
3.2 Discussion

Esculetin is a coumarin derivative (6,7-dihydroxycoumarin; cf. Figure 1-3) and has been categorized under simple coumarins, which are also known as cichorigenin (Kadakol, et al., 2016). Previous studies revealed that the main metabolic reactions of coumarin in Phase I reaction is hydroxylation as catalyzed by hepatic cytochrome P450 (CYP) 2A6 and subsequently further extensively conjugated with D-glucuronic acid and/or sulfate at 7-OH (Egan, et al., 1990; Miles, et al., 1990; Zhu, et al., 2015). A recent study revealed that UGT1A6 and UGT1A9 could mediate 7-O-monoglucuronidation of esculetin (Zhu, et al., 2015). In contrast, the SULT enzymes that are responsible for the sulfation of esculetin, remained unknown. Therefore, the identification of human SULTs involved in the metabolism of esculetin may provide essential information to its pharmacokinetics and toxicokinetics, since the SULTs are known to be involved in the metabolism of numerous xenobiotic compounds (Negata, and Yamazoe, 2000; Chapman, et al., 2004). In this study, the thirteen known human SULTs previously cloned, expressed and purified, were analyzed to reveal those have the capacity to sulfate esculetin. The results obtained from enzymatic assays showed that five of the thirteen SULTs, SULT1A1, SULT1A2, SULT1A3, SULT1B1, and SULT1C4, exhibited sulfating activity toward esculetin. Of these five human SULTs, the sulfating activities of SULT1A2, and SULT1C4 toward esculetin were considerably weaker than those of the other three. SULT1A1 exhibited the strongest sulfating activity toward esculetin, followed by SULT1A3 and SULT1C4 (Table 3.1). Previous studies revealed that both SULT1A1 and SULT1A3 displayed activity toward compounds containing phenolic hydroxyl groups. Therefore, it was expected that SULT1A1 and SULT1A3 have the
capacity to sulfate esculetin since esculetin contains two phenolic hydroxyl groups in its chemical structure (Reiter, and Weinshilboum, 1982; Anderson, and Weinshilboum, 1980). In regard to SULT1C4, although its crystal structure is unavailable, it has been reported to be also capable of sulfating many compounds containing phenolic hydroxyl groups in their chemical structures, including acetaminophen, raloxifene, oxymorphone, naloxone, hydromorphone, p-nitrophenol, 4-aminophenol, 1-naphthol, 2-naphthol, 2-ethylphenol, 4-ethylphenol, 2-n-propylphenol, 2- butylphenol, 4-n-amylphenol, vanillin, p-cresol, bisphenol A, 4-n-octylphenol, and 4-n-nonylphenol (Runge-Morris, and Kocarek, 2013; Yamamoto, et al., 2015). These three latter esculetin-sulfating SULTs have been shown to be expressed in different organs and, possibly, at different developmental stages. For example, SULT1A1 is expressed in the liver, as well as brain, gastrointestinal tract, platelets, and placenta (Barker, et al., 1994), SULT1C4 is expressed in fetal kidney and lung, as well as adult ovary and kidney (Sakakibara, et al., 1998); and SULT1A3 is expressed in jejunum, gastrointestinal tract, platelets, brain and fetal liver (Javitt, at al., 2001).

The pH-dependence studies of the esculetin-sulfating activity of SULT1A1 showed a pH optimum spanning pH 5.5 to 6.5 with maximum sulfating activity detected at pH 5.5-6.5 (cf. Figure 3-1), whereas SULT1A3 exhibited a pH optimum at pH 6.5 (cf. Figure 3-2). SULT1C4 showed a pH optimum spanning pH 6.5 to 8 (c.f Figure 3-3). For further characterization of esculetin-sulfating activity of the kinetic parameters of SULT1A1, SULT1A3, and SULT1C4 were analyzed based on the Michaelis-Menten kinetics with non-linear regression using the GraphPad Prism 5 software program. SULT1A1 showed the lowest $K_m$ (2.229 µM) indicating that the affinity of SULT1A1 for
esculetin is much higher than that of SULT1C4 (34.29 μM) and SULT1A3 (95.49 μM). In terms of $V_{\text{max}}/K_m$, SULT1A1 (14.72) was much higher than SULT1C4 (1.57) and SULT1A3 (0.199), indicating that SULT1A1 is catalytically more efficient in mediating the sulfation of esculetin than SULT1C4 and SULT1A3. It can therefore be concluded that SULT1A1 is likely a major enzyme involved in mediating the sulfation of esculetin in the body.

To investigate further whether esculetin-sulfating activity is indeed present in human organs, cytosol fractions prepared from human lung, liver, kidney, and intestine were used. Previous studies have demonstrated the presence of many SULTs in these four organs (Gamage, et al., 2006). It was therefore not surprising that esculetin-sulfating activity was detected in all four cytosol samples analyzed (Table 3.4). Of these four organ samples, cytosols prepared from human intestine (552.98 pmol/min/mg protein) and liver (261.00 pmol/min/mg protein) exhibited considerably higher esculetin-sulfating activity than the cytosols prepared from human lung (34.45 pmol/min/mg protein) and kidney (42.20 pmol/min/mg protein). These results indicated that intestine and the liver are likely major human organs involved in the metabolism of esculetin through sulfation.

To investigate whether sulfation of esculetin occurs in human cells, cultured HepG2 human hepatoma cells and Caco-2 human intestinal epithelial cells were tested. Previous studies have revealed that the ability of Caco-2 intestinal epithelial cells to express SULT1A1, SULT1A2, SULT1A3, SULT1B1, SULT1C2, SULT1C4, and SULT2A1 and HepG2 human hepatoma cells to express SULT1A1, SULT1A2, SULT1A3, SULT1E1, and SULT2A1 (Teubner, et al., 2007; Westerink, et al., 2007; Riches, et al., 2009; Yamamato, et al., 2015).
In Figure 3-7, autoradiograph of the TLC plate used for the analysis of spent media of HepG2 cells and Caco-2 cells labeled with \([^{35}\text{S}]\)sulfate in the presence of increasing concentrations of esculetin revealed that the generation and release of \([^{35}\text{S}]\)sulfated esculetin in a concentration-dependent manner. Amount of \([^{35}\text{S}]\)sulfated esculetin, indicated by the solid arrow, continued to increase with increasing concentrations of esculetin added to the labelling media. HepG2 cells appeared to generate considerably more \([^{35}\text{S}]\)sulfated esculetin than Caco-2 cells. This finding is in line with previous studies showing that SULT1A1, which showed the strongest sulfating activity toward esculetin, is more highly expressed in the liver than in intestine. Nevertheless, the results indicated that both HepG2 cells and Caco-2 cells were capable of metabolizing esculetin by sulfation.

In conclusion, this study provided clear evidence that human SULT1A1, SULT1A3, and SULT1C4 exhibited sulfating activities toward esculetin among all thirteen human SULTs. Esculetin-sulfating activity was detected in the cytosol or S9 fractions of human intestine, liver, lung, and kidney. Moreover, cultured HepG2 human hepatoma cells and Caco-2 human intestinal epithelial cells were shown to be able to sulfate esculetin under metabolic conditions. These results provided valuable biochemical information for further investigation on the metabolism of esculetin in \textit{vivo}.
References


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