IN VITRO INHIBITION OF HERBAL CONSTITUENTS ON UGT2B7 ENZYME ACTIVITY

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IN VITRO INHIBITION OF HERBAL CONSTITUENTS ON UGT2B7 ENZYME ACTIVITY

by

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage sign
°C	Degree celcius
µL	Microliter
µM	Micromolar
ANOVA	One way analysis of variance
BSA	Bovine serum albumin
cDNA	Complementary DNA
CL_{int}	Intrinsic clearance
$CuSO_4.5H_2O$	Copper(II) sulfat pentahydrate
CV	Coefficient variance
CYP	Cytochrome P450
DMSO	Dimethyl sulfoxide
FDA	United State of Food and Drug Administration
g	Gravity
HDI	Herb-drug interaction
HHI	Herb-herb interaction
HLM	Pooled human liver microsomes
HPLC	High performance liquid chromatography
IC_{50}	Half-maximal inhibitory concentration
ICH	International conference on Harmonization
I.D.	Internal diameter
IS	Internal standard
KCI	Potassium chloride

KH_2PO_4	Potassium dihydrogen phosphate
K_2HPO_4	Dipotassium hydrogen phosphate
K_i	Inhibition constant
K_m	Michaelis-Menten constant
$KNaC_4H_4O_6.4H_2O$	Potassium sodium tartrate tetrahydrate
LLOQ	Lower limit of quantififcation
LOD	Limit of detection
mg	Milligrams
mL	Milliliters
min	Minutes
mM	Milimolar
$MgCI_2$	Magnesium chloride
4MUG	4-Methylumbelliferone glucuronides
Na_2CO_3	Sodium carbonate
NaOH	Sodium hydroxide
pmol	Picomole
QC	Quality Control
RLM	Pooled rat liver microsomes
RME	Relative mean error
rpm	Revolutions per minute
SD	Standard deviations
$SD_{y\text{-}int}$	Standard deviation of y-intercept
Tris-HCL	Tris(hydroxymethyl)aminomethane hydrochloride
UDPGA	UDP-glucuronic acid
UGT	UDP-glucuronosyltransferase

UV/Vis	Ultraviolet/visible
V_{max}	Maximal reaction velocity
WHO	World Health Organization
w/v	weight per volume
Zidovudine	ZDV
Zidovudine glucuronides	ZDVG

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PERENCATAN *IN VITRO* KONSTITUEN HERBA KE ATAS AKTIVITI ENZIM UGT2B7

ABSTRAK

Interaksi herba-dadah boleh menyebabkan induksi dan perencatan enzim metabolisme dadah di mana dapat memberi kesan buruk terhadap badan. Dua jenis enzim metabolisme drug yang utama adalah enzim cytochrome P450 (CYP) dan UDP-glukuronosiltransferase (UGT). Walaupun interaksi herba-drug melalui CYP telah banyak dikaji, interaksi tersebut terhadap UGT masih lagi terhad. Dalam kajian ini, kesan perencatan secara *in vitro* tiga belas konstituen herba bioaktif terhadap salah satu isofom terpenting UGT iaitu UGT2B7 di dalam mikrosom hepar manusia (HLM) dan mikrosom hepar tikus (RLM) telah disiasat. UGT2B7 ialah isofom UGT yang bertanggungjawab untuk konjugasi opioid seperti morfin dan kodeina, ubat antiradang bukan steroid seperti diklofenac dan ketoprofen serta ubat anti-HIV, zidovudin dengan glukuronik asid. Di dalam kajian ini, zidovudin (ZDV) telah digunakan sebagai substrat prob untuk menilai aktiviti isofom tersebut. Konstituen-konstituen herba bioaktif yang telah dikaji potensi perencatan mereka terhadap UGT2B7 adalah andrografolida, arekaidina, arekolina, (+)-katekin, asid galik, kaempferol-3-rutinosida, mangiferin, mitraginina, kuersetin, vanilin, vitexin, isovitexin dan zerumbon. Pembentukan ZDV glukuronida (ZDVG) daripada ZDV oleh HLM dan RLM telah ditentukan menggunakan kaedah kromatografi cecair berprestasi tinggi yang telah disahkan. Parameter kinetik enzim untuk glukuronidasi ZDV iaitu nilai V_{max} dan K_m di dalam HLM adalah sebanyak 1450 ± 26.06 pmol/mg/min dan 0.88 ± 0.05 mM masing-masing dengan nilai perkumuhan intrinsik (V_{max}/K_m) sebanyak 1.65 μL/mg/min. Sementara itu, nilai V_{max} dan K_m di dalam

RLM adalah sebanyak 1089 ± 33.50 pmol/mg/min dan 6.77 ± 0.60 mM masing-masing di mana menghasilkan nilai perkumuhan intrinsik sebanyak 0.16 μL/mg/min. Kajian perencatan di dalam HLM telah menunjukan empat daripada tiga belas konstituen herba yang dinilai telah merencat glukuronidasi ZDV. Zerumbon telah menunjukan perencatan yang paling poten di dalam HLM dengan nilai IC_{50} sebanyak 4.57 ± 0.23 μM diikuti andrografolida, mitraginina and kaempferol-3-rutinosida dengan nilai IC_{50} masing-masing adalah 6.18 ± 1.27 μM, 8.11 ± 4.48 μM and 18.56 ± 8.62 μM. Untuk kajian perencatan di dalam RLM pula, hanya terdapat dua konstituen herba yang telah menunjukan perencatan ke atas glukuronidasi ZDV. Dua konstituen tersebut adalah zerumbon dan mitraginina dengan nilai IC_{50} sebanyak 8.14 ± 2.12 μM dan 51.20 ± 5.95 μM masing-masing. Berdasarkan data kinetik enzim dan potensi perencatan konstituen herba yang telah dikaji, disimpulkan bahawa terdapat perbezaan antara model manusia dan tikus yang telah digunakan. Hasil kajian *in vitro* ini menunjukan bahawa pengambilan zerumbon, andrografolida, mitraginina and kaempferol-3-rutinosida secara serentak dengan drug-drug UGT2B7 boleh menyebabkan interaksi herba-drug berlaku. Walau bagaimanapun, kajian klinikal secara *in vivo* diperlukan untuk mengesahkan hasil kajian *in vitro* ini.

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ABSTRACT

Herb-drug interaction may cause induction and inhibition of drug metabolizing enzymes which may lead to adverse effect in the body. Two major drug-metabolism enzymes in the human body are cytochrome P450 (CYP) and UDP-glucuronosyltransferase (UGT) enzymes. Whilst CYP-mediated herb-drug interactions have been evaluated in numerous studies, the interactions on UGT enzymes are still limited. In the current study, the *in vitro* inhibitory effect of thirteen herbal bioactive constituents on one of the most important UGT isoform which is UGT2B7 in human (HLM) and rat liver microsomes (RLM) had been investigated. UGT2B7 is an UGT isoform that is responsible for conjugation of opioids like morphine and codeine, non-steroidal anti-inflammatory drugs such as diclofenac and ketoprofen and an anti-HIV drug, zidovudine (ZDV) with glucuronic acid. In this study, ZDV was used as the probe substrate for evaluating UGT2B7 activity. The herbal bioactive constituents studied for potential UGT2B7 inhibitory potency were andrographolide, arecaidine, arecoline, (+)-catechin, gallic acid, kaempferol-3-rutinoside, mangiferin, mitragynine, quercetin, vanillin, vitexin, isovitexin and zerumbone. The formation of ZDV glucuronide (ZDVG) from ZDV by HLM and RLM was determined using a validated high performance liquid chromatography method. The enzyme kinetics parameters of zidovudine glucuronidation which are V_{max} and K_m values in HLM were 1450 ± 26.06 pmol/mg/min and 0.88 ± 0.05 mM respectively with an intrinsic clearance value (V_{max}/K_m) of 1.65 µL/mg/min. Meanwhile, the V_{max} and K_m values in RLM were 1089 ± 33.50 pmol/mg/min and

6.77 ± 0.60 mM respectively with an intrinsic clearance value of 0.16 µL/mg/min. Inhibition study in HLM showed that four out of the thirteen herbal constituents evaluated had inhibited zidovudine glucuronidation. Zerumbone showed the most potent inhibition in HLM with an IC_{50} value of 4.57 ± 0.23 µM followed by andrographolide, mitragynine and kaempferol-3-rutinoside with their respective IC_{50} values of 6.18 ± 1.27 µM, 8.11 ± 4.48 µM and 18.56 ± 8.62 µM. For inhibition study in RLM, only two of the herbal constituents showed inhibition on ZDV glucuronidation. They were zerumbone and mitragynine with IC_{50} values of 8.14 ± 2.12 µM and 51.20 ± 5.95 µM respectively. Based on the enzyme kinetics data as well as the inhibitory potency of the constituents evaluated, it can be concluded that there is a difference between human and rat model. This *in vitro* result indicates that consumption of zerumbone, andrographolide, mitragynine and kaempferol-3-rutinoside concomitantly with UGT2B7 drugs may contribute to herb-drug interactions. However, an *in vivo* clinical study is warranted to confirm this *in vitro* finding.

CHAPTER ONE

INTRODUCTION

Herbal medicines are herbal plant-based medical preparations used for prevention and treatment of multiple health conditions have existed for centuries. Discovery of Mesopotamia clay tablets and Egyptian papyrus writings had described the medicinal uses of plants as early as around 3,000 BC. Advancement in modern medicines nowadays is undeniable based on the knowledge of ancient civilization. Most of the modern drugs such as the analgesic morphine, cardiotonic glycoside digitoxin and anti-malarial arteminisin are formerly isolated from herbal plants (Dias et al., 2012). The long tradition of herbal plants as medicinal sources in which the knowledge are inherited from generation to generation has led to the reliability of herbal medicine to be practiced until today. The folks live in the rural area and developing countries extensively use herbal medicines as their primary medications. In addition, majority people in developed countries also use herbal medicines as complementary and/or alternatives medicines (CAMs) to the conventional medicines (Rivera et al., 2013).

WHO (2004) defines herbal medicines as plant-derived materials or preparation with therapeutic or other human health benefits which contain either raw or processed ingredients from one or more plants. The plant-derived materials might be the leaves, flowers, fruit, seed, stems, wood, bark, roots, rhizomes or other plant parts that wholly fragmented or powdered. Herbal medicines have been categorized as food products or dietary supplements in many countries around the world which made their evidence of quality, efficacy, and safety are not required before

marketing. Furthermore, herbal-based products are affordable and assessable which also contribute to their booming usage these days. This raise concerns particularly if the consumers take herbal medicines together with the conventional drugs at the same time or within a short time interval of each other. The practice may give rise to herb-drug interaction (HDI) which is likely to happen among the elderly and chronic diseases patients (Gardiner et al., 2006; Loya et al., 2009).

Herbal medicines contain numerous of constituents in them which may interact with the drugs through pharmacodynamic or pharmacokinetic basis when both are taken concomitantly. A pharmacodynamic interaction results in enhancement (additivity/synergism) or diminishment (infra-additivity/antagonism) of the drug responses when the constituent interferes with the drug's target site, signal or its effector levels. However, the interactions do not change the bioavailability of the drug in plasma or at the targeted site of the action. Meanwhile, pharmacokinetic interactions, on the other hand, occur when the constituent alters the absorption, distribution, metabolism, or elimination of the drug, thereby changing its concentration in plasma and, consequently, at the targeted site of action. Clinically significant HDIs are most frequently due to alterations in pharmacokinetics of the drug, secondary to modulation of drug metabolizing enzymes (Robertson and Penzak, 2006).

Briefly, drug metabolizing enzymes primarily expressed in the liver can be categorized into two phases which are Phase I and Phase II enzymes. Cytochrome 450 (CYP) and UDP-glucuronosyltransferes (UGT) are the most important metabolizing enzymes that catalyze the oxidation reaction in Phase I and

glucuronidation conjugation in Phase II respectively. These enzymes are responsible in increasing the hydrophilicity of lipophilic compounds for facilitating their elimination from the body via urine or bile. Accumulation of the compounds may cause toxicity in the body. Lipophilic compounds will undergo Phase I reactions for introduction or exposure of new functional groups which increases their polarity and hydrophilicity. Even so, Phase I metabolites are often pharmacologically active, chemically reactive or even toxic. To diminish the adverse effects as well as to facilitate excretion from the body, Phase I metabolites will undergo Phase II reactions that conjugate the metabolites with endogenous substances in the body. Some compounds may already have nucleophilic functional groups on them which can directly undergo Phase II reactions without prior Phase I reactions. Induction of drug-metabolizing enzymes will increase the elimination of the therapeutic drugs from the body and causes treatment failure whilst inhibition of the enzymes will decrease the drug elimination from the body and results in toxicity due to drug accumulation (Gibson and Skett, 2001).

HDIs mediated by CYP enzymes have been widely described up to clinical setting (Skalli et al., 2007; Izzo and Ernst, 2009). In contrast, HDIs through glucuronidation reaction have not been effectively studied (Mohamed and Frye, 2011). UGT2B7 is an important enzyme of glucuronidation reaction. It was reported that 35% of the top 200 prescribed drugs in United States undergo UGT2B7 to be metabolized (Williams et al., 2004). UGT2B7 exclusively metabolizes the vital anti-HIV drug zidovudine (Barbier et al., 2000; Boase and Miners, 2002) and majorly metabolizes morphine (Coffman et al., 1997), an analgesic which is widely used in cancer treatment (Bryan, 2018). There are 30-79% of HIV patients (Wiwanitkit,

2003; Littlewood and Vanable, 2008; Bahall, 2017) and 19-53% of cancer survivals (Damery et al., 2011; Liu et al., 2012; Farooqui et al., 2016; Rashrash et al., 2017) take herbal medicines along with the conventional drugs. Hence, the probability of herbal medicines intake with the drugs metabolized by UGT2B7 i.e. zidovudine and morphine is exist and the study of herb-drug interactions via UGT2B7 becomes critical.

Although all herbal constituents have the potential to modulate the drug metabolizing enzymes (DMEs), it is vital to investigate the effect of herbal bioactive constituents on DMEs. Herbal bioactive constituents are the ones that are involved in the therapeutic mechanism of action such as anticancer, antimicrobial, antioxidant, analgesic and wound healing activity of the herbal plants (Sasidharan et al., 2011). In this study, thirteen herbal bioactive constituents were investigated for their inhibitory effects on UGT2B7 in human and rat liver microsomes. Zidovudine was used as the probe substrate to monitor the UGT2B7 isoform activity. These *in vitro* studies were analyzed using a modified and validated high performance liquid chromatography (HPLC) method. Meanwhile, human and rat liver microsomes were used to investigate interspecies differences as rats are the most common pre-clinical animals utilized *in vivo*. Data generated in this study will provide useful information for researchers with an intention to carry out further *in vivo* study in rats and humans as well as to provide a better understanding of the risks associated with the concomitant use of herbal medicines with pharmaceutical drugs among the general public including healthcare professionals.

1.1 Objectives of the Study

The objectives of this thesis are listed below:

i) To modify and validate an HPLC method for quantification of zidovudine glucuronides in liver microsomes.

ii) To establish optimized conditions of zidovudine glucuronidation assay in human (HLM) and rat liver microsomes (RLM).

iii) To determine the inhibitory effect of andrographolide, arecoline, arecaidine, (+)-catechin, gallic acid, kaempferol-3-rutinoside, mangiferin, mitragynine, quercertin, vanillin, vitexin, isovitexin and zerumbone on zidovudine glucuronidation in HLM and RLM respectively.

CHAPTER TWO

LITERATURE REVIEW

2.1 Xenobiotics Metabolism

Xenobiotics are the non-nutrient foreign substances that may enter human body through ingestion, inhalation or absorption such as the pharmaceutical drugs, antibiotics, food additives, environmental pollutants and pesticides. Once xenobiotics enter the body, they need to be eliminated out from the body or else they will accumulate and cause toxicity. There are three primary ways of xenobiotics elimination from the body namely renal excretion, biliary excretion and metabolism. Hydrophilic and polar compounds are eliminated by renal and biliary excretion whereas lipophilic compounds tend to be reabsorbed back into the systemic circulation which can cause their accumulation in the body. To prevent this occurrence, metabolism reactions will transform the lipophilic compounds into more polar and hydrophilic compounds which then facilitate their elimination via urine and bile. The metabolites formed are mostly less toxic than the parent compounds. As most of the xenobiotics are lipophilic compounds, metabolism process plays a critical role in xenobiotics detoxification and elimination. Furthermore, Williams et al. (2004) reviewed that three quarters of the top 200 prescribed drugs in United States undergo metabolism as the clearance mechanism followed by the renal and biliary excretion.

Xenobiotics metabolism can be classified into Phase I and Phase II metabolisms. Both phases are catalyzed by a group of specialized enzymes. Phase I metabolism comprises of functionalization enzymes that catalyze oxidation, reduction and hydrolysis reactions resulting in the addition or revealing of a functional group such as –OH, -SH, -NH2, -COOH on the substrate (Gonzalez et al., 2011). Oxidation reaction is the most common reaction in Phase I metabolism compared to the other reactions which is carried out by cytochrome P450 (CYP), flavin-containing monooxygenase (FMO) and epoxide hyrolases (EH). Furthermore, oxidation by cytochrome P450 (CYP) enzymes is responsible for 75% of marketed drugs (Williams et al., 2004). If the metabolites of phase I metabolism are sufficiently polar, they may be readily excreted at this point. However, many products of phase I metabolism are not eliminated rapidly and undergo a subsequent reaction of the phase II. In Phase II metabolism, its group of enzymes catalyzes the conjugation of the substrates with polar endogenous substances such as glucuronic acid, amino acid, gluthathione and S-adenosylmethionine. Examples of such enzymes are UDP-glucuronosyltransferase (UGT), sulfotransferase (SULF), gluthathione S-transferase (GST) and methyltransferase (METH) which catalyze the glucuronidation, sulfation, gluthathione and methylation conjugation reactions respectively. Conjugation reactions are generally known as true detoxification reactions as the metabolites formed are usually pharmacologically inactive and high in polarity. It is essential to be noted that a compound can undergo Phase II reaction without prior Phase I reaction (Gibson and Skett, 2001).

Metabolizing enzymes are primarily expressed in the liver which is located between the portal vein and the inferior vena cava in the body. Portal vein transports

blood from gastrointestinal tract to the liver meanwhile inferior vena cava drains blood from the liver to the heart for circulation throughout the body (Holt and Smith, 2008). Therefore, any oral administration of xenobiotics (e.g. drugs, food additives, poisons and antibiotics) will be metabolized by the liver before circulation to the whole body. Due to the metabolism, only a small portion or almost none of the xenobiotics will reach the blood circulation whereas the rest will be in the form of their less potent metabolites. This is an example on how metabolism protects the body from xenobiotics accumulation that may cause toxicity. Metabolizing enzymes are also prevalent in other extrahepatic organs and tissues including gastrointestinal tract, small intestine, kidney and brain (Jhajra et al., 2011). At the subcellular level, metabolizing enzymes are located in the smooth endoplasmic reticulum and cytosol (Correia, 2004). In addition, administration of xenobiotics may alter the metabolizing enzymes. The amount of enzymes produced will increase if the enzymes are induced, thus the rate of drug metabolism will be increased. In the situation where two substances compete for the same enzyme, enzyme inhibition is known to take place. As a consequence, the rate of drug metabolism will be reduced. The inducers, inhibitors or xenobiotics may be substrates for different drug metabolizing enzymes (Tredger and Stoll, 2002).

2.2 UDP-Glucuronosyltransferases

The uridine 5'–diphospho-glucuronosyltransferases (UGTs) are a family of membrane-bound enzymes of endoplasmic reticulum primarily found in the liver. These Phase II enzymes are accountable for the reaction known as glucuronidation. Glucuronidation is a major route in Phase II metabolism for detoxifying and elimination of xenobiotics from the body (Meech and Mackenzie, 1997; Alkharfy

and Frye, 2007). Wells et al. (2004) reviewed that an approximately 40-70% of clinical drugs in human are metabolized through this pathway. Nevertheless, endobiotics such as bilirubin, steroids, retinoids and bile acids also undergo this route of detoxification (King et al., 2000). In glucuronidation, UGT enzymes catalyze the covalent addition of glucuronic acid from UDP-glucuronic acid (UDPGA) to the nucleophilic functional site of O-, N-, S-, or C- presence on a substrate. The generated products are the water soluble glucuronides and UDP. Glucuronidation reaction is depicted in Figure 2.1.

The UGT superfamily can be grouped into four families namely UGT1, UGT2, UGT3 and UGT8. The nomenclature for UGT enzymes starts with Arabic numeral which denotes the family (e.g. UGT1) followed by a letter which represents the subfamilies (e.g. UGT1A), and finally the second Arabic numeral which designates the individual genes (e.g. UGT1A1). For each family, the enzymes share no less than 40% homology in their DNA sequences whereas in each subfamily, the enzymes shares at least 60% homology in their DNA sequences (Mackenzie et al., 2005). Among the four UGT families, only UGT1 and UGT2 use UDPGA as sugar donor for glucuronidation reactions. They play the critical roles in metabolizing endogenous and exogenous compounds as aforementioned. In contrast, UGT3 family which consist of UGT3A1 and UGT3A2 use, respectively, UDP-N-acetylglucosamine and UDP-glucose/UDP-xylose to conjugate bile acids, steroids and bioflavones (Meech et al., 2012). Meanwhile, the sole UGT8 family member UGT8A1 utilized UDP-galactose to conjugate galactosidate ceramide and bile acids (Meech et al., 2015). The UGT1 family identified at present consists of nine functional isoforms in human (UGT1A1, UGT1A3-UGT1A10) (Ritter et al., 1992;

Mackenzie et al., 1997) and seven in rats (UGT1A1-UGT1A3, UGT1A5-UGT1A8) (Emi et al., 1995). Meanwhile for UGT2 family, it includes six known isozymes in humans (UGT2B4, UGT2B7, UGT2B10, UGT2B11, UGT2B15 and UGT2B17) and seven in rats (UGT2A1, UGT2B1, UGT2B2, UGT2B3, UGT2B6, UGT2B8 and UGT2B12) (Ritter, 2000).

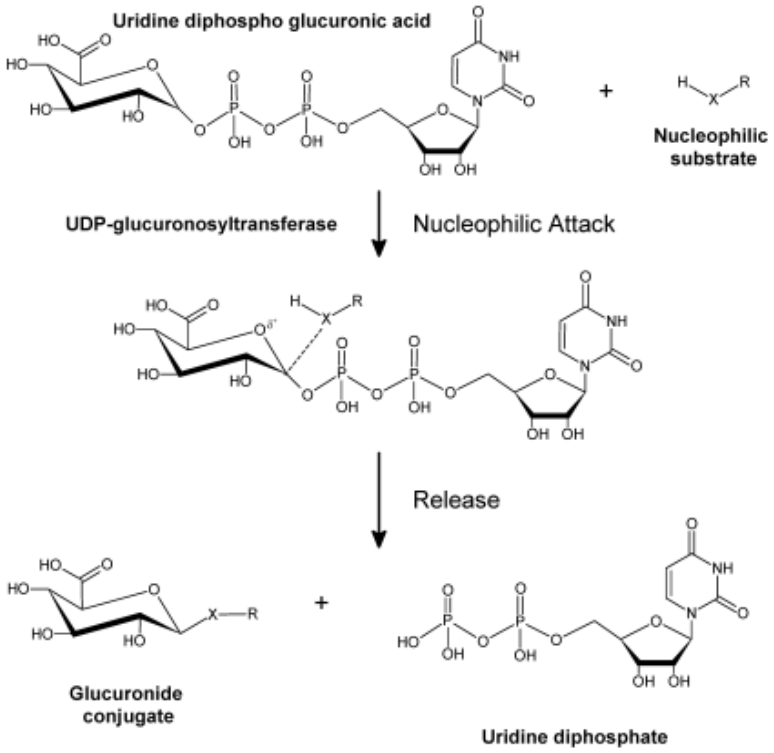

Figure 2.1: Glucuronidation reaction scheme (Rowland et al., 2013).

2.3 UGT2B7 Isoform

UGT2B7 appears to be a very important human UGT isoform. It catalyzes almost 35% of the top 200 prescribed drugs in United States, which also ranked first among the other UGT isoforms (Williams et al., 2004). The drugs that have been identified to be metabolized by this isoform are the opioids such as morphine and codeine

(Coffman et al., 1997), the non-steroidal anti-inflammatory drugs (NSAIDs) include the diclofenac, niflumic acid and mefanamic acid (Mano et al., 2007a), the anticonvulsant valproic acid (Jin et al., 1993) and the therapeutic agent used in HIV treatment, zidovudine (Barbier et al., 2000). The hydroxylated metabolites of 2-acetylaminofluorene and benzo[α]pyrene also undergo UGT2B7 to be eliminated out from the body (Jin et al., 1993). 2-acetylaminofluorene is carcinogens used in carcinogenesis study meanwhile benzo[α]pyrene is the environmental pollutants formed during incomplete combustion or pyrolysis of organic material. Both are harmful non-drug xenobiotics that might be exposed to human through inhalation or dermal contact. In addition, UGT2B7 also generated the toxic metabolites such as the highly cholestatic estradiol-17-glucuronides (Vore et al., 1983) and the protein-bound acyl-glucuronides of certain NSAID such as diflunisal which elicit toxic immunological responses (Worrall and Dickinson, 1995). Besides all these xenobiotics metabolism, this isoform also participates in the catalyzation of several physiologically endogenous compounds such as steroid hormones, bile acids, retinoids and fatty acids (Holthe et al., 2003).

Despite the significant role plays by UGT2B7 isoform in elimination and detoxification of a variety of compounds, the structural features of its substrates selectivity are still poorly understood. This is because most of the UGT isoforms usually possess distinct, but overlapping substrates and inhibitors selectivities. This can be seen in the glucuronidation of estradiol where the UGT1A1 isoform catalyze its 3-*O*-glucuronidation while UGT2B7 isoform catalyzes the 17-*O*-glucuronidation which generated estradiol-3-glucuronides and estradiol-17-glucuronides respectively (Alkharfy and Frye, 2002; Pfeiffer et al., 2005). In addition, UGT2B7 glucuronidates

morphine into morphine-3-glucuronides and morphine-6-glucuronides, displaying that a single UGT isoform can also catalyze a substrate at different positions (Stone et al., 2003). Meanwhile, the *in silico* study conducted by Sorich et al. (2006) described that UGT2B7 tends to catalyze substances that possess functional group of phenolic, carboxylic acid, aliphatic hydroxyl, aliphatic amine and aromatic nitrogen even though the catalyzation is still overlapping with the other UGT isoforms.

UGT2B7 is primarily expressed in the liver even though it also shows expression in the gastrointestinal tract, kidney, pancreas and brain (Radominska-Pandya et al., 2001). The UGT2B7 gene is localized within a cluster of UGT2B genes at chromosomal 4q13. This isoform has been demonstrated to exist as homodimers protein with two equivalents, interacting substrate binding sites along with the other multiple effector sites. Due to this, different UGT2B7-catalyzed substrate will produce different kinetics data (Uchaipichat et al., 2008; Lewis et al., 2011). As described by Uchaipichat et al. (2008), UGT2B7-catalyzed zidovudine (ZDV) glucuronidation in human recombinant enzyme follows hyperbolic (Michaelis-Menten) kinetics whereas the catalyzation of 4-methylumbelliferone (4MU) and 1-naphtol (1NP) possess the positive homotropic cooperativity, a situation where the binding of second substrate molecule increases the substrate affinity towards the enzyme. Interestingly, ZDV glucuronidation kinetics changed from hyperbolic (Michaelis-Menten) to sigmoidal curve in the presence of either 1NP or 4MU as the inhibitor which suggested the heterotropic cooperativity. Despite that, the homotropic cooperativity of 4MU and 1NP was unaltered in the presence of ZDV even though their glucuronidation rates were decreased. In addition, the result also shows that 1NP inhibited 4MU glucuronidation whilst 4MU activated 1NP

glucuronidation in UGT2B7 isofom. These data clearly suggest that UGT2B7 dimerization exhibits distinct effector site along with the substrate binding sites as mentioned.

Moreover, UGT2B7 may also form the heterodimers between the other UGT isoform such as UGT1A1 and UGT1A9 (Yuan et al., 2016). It has been proposed that, the UGT isoform heterodimers may allow the formation of a new substrate-recognition site which do not applied to the homodimers (Bock and Köhle, 2009). However, the inter-isoform hetero-dimerizing interactions are obviously weaker. Therefore, it has been suggested that the stable homodimers of UGT2B7 are predominantly *in vivo* (Yuan et al., 2016).

A number of studies had shown that different isoforms in different species may catalyze similar classes of compounds as human UGT2B7 did. Previous studies revealed that the formation of morphine-3-*O*-glucuronide is catalyzed by UGT2B7 in human (Turgeon et al., 2001) and UGT2B1 in rat (Pritchard et al., 1994). It is well known that rat UGT2B1 is a recognized orthologue of human UGT2B7 (King et al., 2000; Ritter, 2000; King et al., 2001). In addition, monkey UGT2B9 and dog UGT2B31 respectively are also suggested to be the simian and canine equivalent of human UGT2B7 (Green et al., 1997; Soars et al., 2003). Animal species such as rat, dog, or monkey is often used in toxicology and pharmacology studies aimed at pharmacodynamics, metabolism, pharmacokinetics, and safety of new chemical entities (NCEs). Therefore, it is crucial to evaluate the interspecies differences in glucuronidation particularly involving UGT2B7 isoform. The *in vitro* interspecies

data obtained is essential for choosing the best pre-clinical animal model to be conducted *in vivo* which further may be extrapolated to human.

2.4 *In vitro* Models for Investigating Drug Metabolism

In vitro model refers to an assay which is carried out outside the living organism by using components isolated from it. The condition used in the assay mimics the isolated component's biological environment. *In vitro* assay is a method of choice in drug metabolism study due to its simplicity and low cost. It produces quick results and less time consuming as compared to *in vivo* study using living organism. Hence, *in vitro* assay is extensively used to determine the inhibition and induction of a drug metabolizing enzyme if two different compounds are employed together. There are several different *in vitro* models frequently used in drug metabolism studies such as microsomes, recombinant expressed enzymes, and hepatocytes systems.

2.4.1 Microsomes

Microsomes are subcellular fractions of endoplasmic reticulum (ER) prepared from differential centrifugation of the homogenated tissues. Briefly, the homogenate tissues are centrifuged around 10,000-18,000*g* to sediment out most of the organelles in the tissues whereby the soluble enzymes and fragmented ER remain in the supernatant. ER is then precipitated out at approximately 100,000*g* of centrifugation leaving the soluble enzyme to remain in the solution. The resulting pellet is the microsomal fraction (Heinemann and Ozols, 1998; Graham, 2002).

Microsomes contain almost only CYP450 and UGT enzymes. These enzymes are the membrane-bound proteins localized in the ER of liver cells. However, to carry out any metabolism reactions using microsomes, cofactors must be added into the assay. While the active site of CYPs locates in the cytoplasmic side of ER, the active site of UGT proteins locates in the luminal side of ER as illustrated in Figure 2.2 (Shepherd et al., 1989; Ishii et al., 2010). It has been suggested that the existence of active transporters in living system is responsible to translocate the substrate and cofactor from cytosolic space into the lumen where UGT active site reside. However, the transporters are not present in microsomes. This causes microsomal glucuronidation to become latent and slower than it should be. The latency phenomenon is thought to be due to the ER membrane obstructing the access of the substrate and cofactor towards the enzyme. Therefore, in microsomal UGT assay, an activation step which disrupt the membrane is required to permit the flow of them into the lumen (Ishii et al., 2012). There are several treatments to activate the microsomal glucuronidation include the uses of detergent, pore-forming alamethicin and sonication.

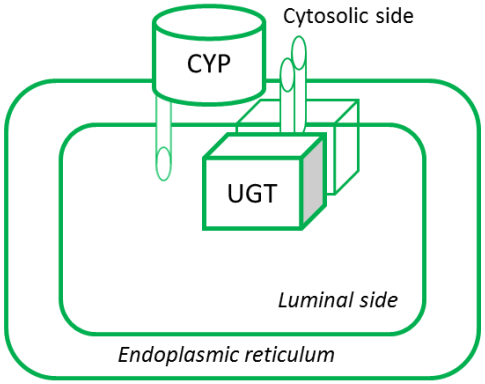

Figure 2.2: The topological model of CYP and UGT in the endoplasmic reticulum (Ishii et al., 2010).

Moreover, microsomes isolated from human and variety of animal species (e.g. rat, dog, and monkey) as well as isolated from various tissues (e.g. liver, kidney, brain and intestine) are commercially available and well characterized. Therefore, microsomes make the interspecies studies in drug metabolism become easier. Comparison of metabolic profiles in different animal species with human is essential in NCEs development studies. The comparison assists in identification of the most relevant animal model for further pharmacokinetics and toxicological studies as aforementioned (Jia and Liu, 2007). Besides that, microsomes are also affordable, have long-term stability during cryopreservation, and easy to prepare which make them as one of the most common tools involved in metabolism studies.

2.4.2 cDNA-Recombinant Expressed Enzyme

Advancement in genetic engineering enables a single metabolizing enzyme isoform to be expressed in a variety of recombinant systems for research purposes. These systems can be divided into four main groups based on the host cell types which are bacteria-based, yeast-based, insect cell-based and mammalian cell-based system (Mallet, 2004). In particular, recombinant system involves transfecting host cells with a DNA vector that contains the protein template and then culturing the cells so that they transcribe and translate the desired protein. Typically, the cells are then lysed to extract the expressed protein for subsequent purification. In addition, this singly expressed enzyme system is extensively used to investigate the enzyme-specific metabolism of a compound as well as to evaluate the inhibitory effects of potential inhibitors towards a specific substrate of an enzyme. The system can also be a useful system to confirm results obtained with microsomes or native human liver tissue. The most common model used in drug metabolism studies is the

baculovirus-insect cell expression system which is also commercially available (Chen et al., 1997; Patten, 2006).

2.4.3 Primary Hepatocytes

Primary hepatocytes are the parenchymal cells of the liver which are isolated directly from the liver tissues. Once isolated, hepatocytes can be held in suspension, in which case they remain viable for only a few hours, or they can be maintained in monolayer culture for a maximum of 4 weeks (Brandon et al., 2003). As an intact cell, primary hepatocytes contain all the metabolizing enzymes, cofactors and transporters of metabolism reactions which maintained at physiological levels. Human primary hepatocytes are therefore considered the preferred *in vitro* model for metabolism study that offers the most resemblance model to the *in vivo* human liver. They are able to mimic the metabolic profile of a compound very similar to that found in the *in vivo* study (Gomez-Lechon et al., 2003). While only inhibition of metabolizing enzymes evaluation can be investigated using microsomes and recombinant expressed enzymes, primary hepatocytes offer both induction and inhibition evaluation.

Despite that, the human liver supply is quite limited and restricted. The supplied liver also may have the pathological status and exhibit inter-individual variation. However, the latter problem can be solved by pooling the primary hepatocytes. In addition, primary hepatocytes do not proliferate *in vitro* and therefore cell cultures need to be prepared each time from the liver tissues which are laborious (Ferrini et al., 1997; Guillouzo et al., 1997; Gomez-Lechon et al., 2004).

These disadvantages had hindered the widespread use of human hepatocytes. Nevertheless, several protocols have been created to cryopreserve human hepatocytes for long-term but still retain the metabolic activity and differentiated phenotype (Mitry et al., 2002). Due to cryopreservation techniques, human primary hepatocytes are commercially available for research purposes (Hengstler et al., 2000).

2.5 Enzyme Kinetics

Enzymes are protein catalysts that enhance and speed up the rate of a chemical reaction without being used up in the process. Meanwhile, enzyme kinetics is the study of enzyme activity in catalyzing a chemical reaction. Michaelis-Menten model proposed in 1913 is one of the simplest and best-known models to evaluate the enzyme kinetics. The model suggested an equation where a substrate binds reversibly to an enzyme to form an enzyme-substrate complex, which is then converted to generate a product and to regenerate the enzyme. This model includes the assumption that at early stage of the reaction or at initial rate velocity of the reaction, so little product is formed and the reverse reaction can be ignored as shown in Equation 2.1. If initial rate velocity is not considered, the enzyme-substrate complex will reversibly generate the product and enzyme as shown in Equation 2.2:

$$E + S \rightleftharpoons ES \rightarrow E + P] \qquad \text{(Eq. 2.1)}$$

$$E + S \rightleftharpoons ES \rightleftharpoons E + P] \qquad \text{(Eq. 2.2)}$$

where E is the enzyme, S is the substrate, ES is the enzyme-substrate complex, and P is the product. Michaelis and Menten discovered that by using initial rates conditions in enzyme kinetic assay, they can avoid the problems due to substrate depletion, product accumulation and progressive inactivation of the enzyme which can then lead to easier experimental data interpretation (Cornish-Bowden, 2013). Initial rates condition is explained by using Figure 2.3. Based on the figure, the amount of product formed at fixed enzyme concentration increases with time at the initial period. However, after the period, the formation starts to reach a plateau even when the time is increased. The same trend is also observed when another substrate concentration was used where the higher concentration approaches plateau faster. The initial velocity (V_o) for each concentration is determined from the slope of the curve. This plateau case occurs as the reaction equilibrium has been attained due to enzyme-saturated with substrate as reaction proceeds. Therefore, rate of product formation is equal to the rate of substrates formation (reverse reaction) as shown in Equation 2.2. This situation can be avoided if the enzyme assay is conducted under initial rate condition as proposed by Michaelis and Menten. Under the condition, the product formation is proportional (linear) with time as the enzyme is not saturated by the substrates yet. In addition, any differences in product formation particularly due to enzyme inhibition can also be easily detected under this condition. Incubation time and protein content are the two parameters that can be optimized to achieve the state of initial rate. In practice, US Food and Drug Administration has suggested no more than 10-30% of product formation should be measured to achieve initial rate condition (FDA, 2006).

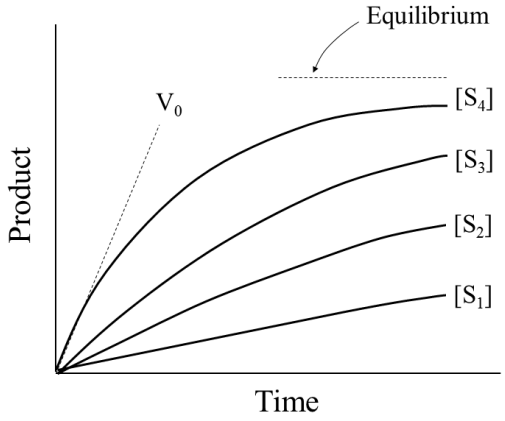

Figure 2.3: The formation of product as a function of time at different substrate concentration. The initial velocity (V_0) for each substrate concentration is determined from the slope of the curve at the beginning of a reaction, when the reverse reaction is insignificant. $[S_1]$, $[S_2]$, $[S_3]$, and $[S_4]$ are the substrate concentrations start from the lowest to the highest concentration (Berg et al., 2002).

Moreover, steady-state assumption proposed by Brigg and Haldene in 1925 assumes that concentration of transition complex [ES] is unchanged over the time period of the assay ($d[ES]/dt = 0$) even if the concentrations of substrates and products are changing. This means that the rates of formation and breakdown of the ES complex are equal throughout the assay as shown in Figure 2.4. The assumption is valid if concentration of substrate presence is higher than the concentration of the enzymes (Cornish-Bowden, 2013).

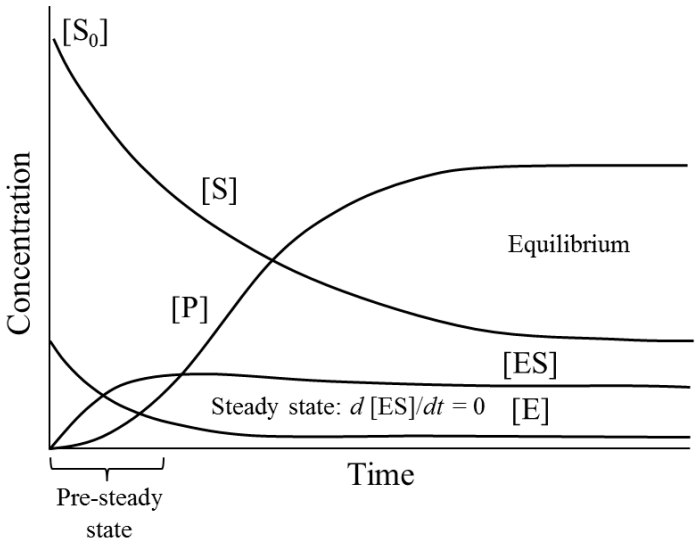

Figure 2.4: Changes in the concentration of reaction participants of an enzyme-catalyzed reaction with time (Berg et al., 2002). $[S_0]$ is the initial substrate concentration, [S] is the substrate concentration, [E] is the enzyme concentration, [ES] is the enzyme-substrate complex concentration, and [P] is the product concentration.

Michaelis-Menten equation under steady-state assumption is used to derive the enzyme kinetic parameters which are V_{max} and K_m values. V_{max} is the maximal velocity of a reaction attained when the catalytic sites on the enzyme are saturated with substrate whereas K_m is the substrate concentration at which the reaction rate is half its maximal velocity value. Therefore, K_m is the concentration of substrate at which half the active sites are filled. For many enzymes, the reaction velocity varies with the substrate concentration [S] in a manner shown in Figure 2.5. The rate of catalysis rises linearly as substrate concentration increases and then begins to level off and approach a maximum at higher substrate concentrations. In enzyme kinetics assay, substrate concentration at or around K_m value is used. This is because when $[S] \leq K_m$, the rate of reaction is directly proportional to the substrate concentration. Thus, the substrate is converted into the product without any reverse reaction

(product converts into substrate) takes place. Meanwhile, when [S] > K_m, the rate of reaction is maximal and independent of substrate concentration which leads to the occurrence of reverse reaction (Berg et al., 2002). Above all, a valid enzyme kinetic assay should be conducted under initial rate condition at or below the K_m value. This also applied to the UGT enzyme.

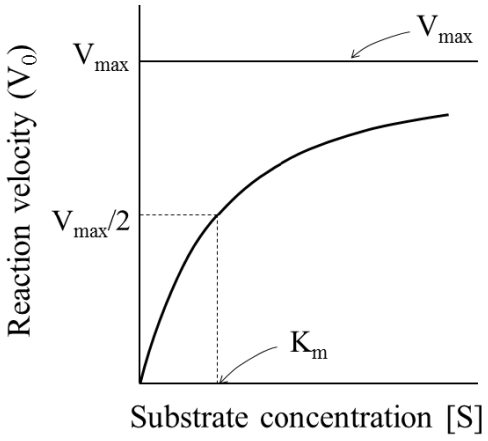

Figure 2.5: Michaelis-Menten Kinetics. A plot of the reaction velocity as a function of the substrate concentration [S] for an enzyme that obeys Michaelis-Menten kinetics shows that the maximal velocity (V_{max}) is approached asymptotically. The Michaelis constant (K_m) is the substrate concentration at enzyme velocity of $V_{max}/2$ (Berg et al., 2002).

2.6 Herbal Medicines and Supplements

Herbal medicines are defined as a plant derived material or preparation with therapeutic or other human health benefits which contain either raw or processed ingredients from one or more plants (WHO, 2004). They have been used for medicines purposes to treat various ailments and diseases since millennia. The first documented records of herbal medicines use date back 5,000 years in China (Rivera et al., 2013). Similarly, Indian's Ayurvedic medicine tradition is thought to be more than 5,000 years old and herbal medicines remain as essential component of its practice (Garodia et al., 2007). The long history traditional use of herbal medicines

has made their safety and effectiveness acknowledged and may be accepted by national authorities. St. John's wort is one of the most popular herbal medicines used throughout the world as antidepressant besides milk thistle which is clinically used due to its hepatoprotective properties (Abenavoli et al., 2002; Rodriguez-Landa and Contreras, 2003). Besides that, it is noteworthy that herbal medicines contains myriad of constituents which work synergistically to produce the therapeutics effects. Some of the constituents also have been developed into modern medicines, either in the natural form such as the analgesics morphine which is isolated from *Papaver somniferum L.* (opium poppy) or as a lead compound subjected to optimization by synthetic organic chemistry such as aspirin (acetylsalicylic acid) which is derived from salicylic acid isolated from willow tree *Salix alba L.* (Dias et al., 2012).

Eventhough there are modern medicines which are proven clinically effective nowadays, herbal medicines remain widespread around the globe with estimation of global herbal market is approximately US $83 billion annually (Robinson and Zhang, 2011). In addition to this, nearly 70-95% of the world population used herbal medicines for primary medicaments particularly in the developing countries where the cost of modern medicines are too expensive for them (Robinson and Zhang, 2011). The use of herbal remedies has also been widely embraced in many developed country such as Europe, North Africa and Australia with the growing utilization of complementary and alternative medicines (CAMs) in the countries (Calapai, 2008; Braun et al., 2010; Anquez-Traxler, 2011). The scenario also can be seen in Malaysia, a country that rich with various plant species. It has been reported that 1,082 species (15%) of angiosperms from more than 20,000 species and 76 species (13%) of ferns from 600 species in Malaysia are possess the therapeutic effects

(Yaacob et al, 2005). Therefore, herbal industry is among the largest contributors to Malaysia economy where the herbal medicines are also widely consumed by its citizens. Malaysian herbal market is projected to reach RM32 billion in the year 2020, with the annual growth between 8-15%. In fact, all Malaysian Development Plans starting from the Third Malaysian Plan to the Tenth Malaysian Plan have included policies related to herbal industry in them such as National Agro-Food Policy (NAFP) and National Key Economic Area (NKEA). It was emphasized that under NAFP and NKEA, the high quality herbal products will be developed and produced for food and beverages industry as well as nutraceutical industry (Zakaria, 2015).

Herbal medicines products are mostly classified as food or dietary supplements in many countries which therefore are not regulated like the pharmaceutical drug. As such, evidence of quality, efficacy, and safety of these herbal medicines are not required before marketing which cause the large production of herbal supplements by the industries (Ekor, 2014). The herbal supplements produced also may either be registered or not with the health authorization. Accordingly, herbal supplements are broadly available not only in the drug stores but also in food stores, supermarkets and even over the internet which give an easy access to the consumers these days. Various advertisements in the mass media including television and radio programs as well as media social (e.g. facebook and instagram) have also contributed to the increasing of the herbal supplements demand among the community.