Investigation into the Roles of Hepatic Uptake Transporters in the Enterohepatic Recycling of Phenolic Glucuronides

By

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ABSTRACT

Drug in vivo exposure decides the drug efficacy. Drug in vivo exposure depends on different ways of drug disposition. Enterohepatic recycling (EHR) serves as an important drug disposition process, which could increase drug in vivo exposure by facilitating the recycle and reabsorption of a drug. The enterohepatic recycling of parent compounds was well studied, but the recycling process of the metabolites were barely investigated. Our recent study indicated that Phase Ⅱ metabolites (glucuronides) could be recycled efficiently as well as their aglycones.

Methods: To study the mechanism of the EHR of the glucuronides, a hepatic portal vein infusion model and a small intestine perfusion model were established on Wistar rat. A cell uptake study was conducted on 3 OATP over-expressed cell lines to elucidate the uptake mechanism of glucuronides. To analyze all the biological samples, an LC/MS method was established and validated.

Results: Our results indicated that glucuronides were able to participate in the EHR as well as their aglycones. Up to 90% of glucuronides could be recovered from bile after infused to liver. OATPs played a significant role in the EHR of glucuronides. The cell uptake results and recycle ratio fitted with substrate-inhibition model, which indicated that hepatic uptake is the-rate limiting step in EHR of glucuronides.

Conclusion: There is another recycling pathway different traditional understanding of enterohepatic recycling. We termed it as “hepatoenteric recycling”, where the roles of metabolism organ and recycling organ are switched. The study would complement the general understanding of enterohepatic recycling and help give a more accurate prediction of drug in vivo efficacy.

Key words: Phenolic compounds; Glucuronide; Enterohepatic recycling; Hepatic uptake

III. TABLE OF CONTENTS

Chapter 1 Introduction

Drug efficacy is directly decided by drug in vivo exposure. With higher in vivo exposure, the drug efficacy is correspondingly higher. Drug in vivo efficacy is influenced by different process in absorption, distribution, metabolism and elimination steps. There are many mechanisms considered having impact on drug in vivo exposure. Enterohepatic recycling/circulation was one of the most important drug disposition process, which could significantly change drug in vivo exposure.

Enterohepatic circulation/recycling could greatly increase drug in vivo exposure through reabsorption process. In general, drugs will go through Phase I or Phase II metabolism (or both) when they are absorbed in small intestine. With the participation of Phase I (CYP 450) and Phase II (mostly UGTs and SULTs) enzymes, drugs will lose their pharmacological activity and biotransformed to Phase II conjugates like glucuronides, sulphate or glutathione conjugates. These conjugates are further excreted into bile by efflux transporters and drugs are able to return small intestine in the form of metabolites through bile secretion. With the help of microflora in down-stream of intestine and colon, some of the metabolites are hydrolyzed and converted back to their aglycone forms. Then, they will have chance to be absorbed again in intestinal tract.

The study of enterohepatic circulation initiated back to 1950s. Over 90% of carbon-4 labeled cholesterol was found to be excreted to intestinal tract. However, rate of appearance of the cholesterol in bile (around 50% in 60-70 hours) was shorter than those appears in feces (120-170 hours). The phenomenon indicated that the

cholesterol went through a circulation before they were eliminated from body [1]. Bile salts and acids were then found to go through an extremely efficient excretion or reabsorption in liver and small intestine. [2] The enterohepatic circulation of bile acids has been studied in 1960s-70s. More than 99% of the bile acids were found to be within the enterohepatic circulation at all times [3]. The circulation enables the consumption of the bile acids go beyond the daily synthesis limit of liver. Other endogenous compounds including bilirubin [4], thyroid hormones and sexual hormones (estrogens and steroids) also go through EHC [5-8].

Except for these endogenous compounds, the enterohepatic recycling of drugs is also worth noticing. The recycling of drugs are slightly different from endogenous compounds. Endogenous compounds are “circulated” in the system, for they remain the active forms and circulate in liver and intestinal tract. However, drugs normally lose their activity and are converted to Phase II metabolites (glucuronides, sulfates etc.). These inactive metabolites are further excreted into bile, recovered in small intestine and restored their pharmacological activity by hydrolysis in intestinal tract. The recovered aglycones are absorbed and recycled in the system. A lot of commonly used drugs undergo some degree of enterohepatic recycling. These including NSAIDS (nonsteroidal anti-inflammatory drugs) like aspirin, indomethacin [9, 10],antimicrobials like rifampicin, statins like pravastatin [11].

The enterohepatic recycling is a comprehensive process requiring the corporation of hepatic uptake, hepatic metabolism, biliary excretion, sometimes gut flora hydrolysis and reabsorption in distal intestine. The uptake and efflux process require the participation of transporter proteins.

The hepatic uptake of bile acids consists of three major mechanisms: sodium-dependent uptake, sodium-independent uptake and passive diffusion [12]. Sodium/taurocholate co-transporting polypeptides (NTCP) were the sodium dependent transporters responsible for the uptake of bile acids. The first Ntcp was cloned from rat liver. NTCPs play a predominant role in the uptake of conjugated bile acids. Less than half of unconjugated bile acids were transported by NTCPs as well. Not only were bile acids, some statins found to be transported by NTCPs [13, 14]. On the other hand, OATP family is responsible for the sodium-independent uptake of bile acids. Most of unconjugated bile acids were transported by OATPs [15].

OATPs are also responsible for the hepatic uptake of endogenous compounds like bilirubin, estrogen hormones and numerous drugs. Several OATPs play a major role in the hepatic uptake of drugs. OATP 1B1, 1B3 and 2B1 are the most abundant transporters expressed in liver. OATP1B1, 1B3 are the first and second identified liver-specific OATP members [16]. They shared over 80% of substrate list and OATP 1B3 might work as a back-up system for OATP 1B1. Commonly used drugs including statins, morphine and antibiotics are all transported via OATP 1B1/1B3. Compared to OATP 1B1/1B3, OATP 2B1 is slightly different. It is ubiquitously expressed throughout body. Except for basolateral side of hepatocyte, it is also expressed in small intestine, kidney, heart, blood-brain barrier and placenta [16]. Some commonly used drugs like statins are also substrate of OATP 2B1. OATP 2B1 is prone to have lower affinity to its substrate and higher capacity. Other uptake transporters including organic cation transporters (OCT) and organic anion transporters (OAT) also participate in the hepatic uptake process. OCT1 and OCT3 are expressed in the basolateral membrane of hepatocyte [17]. OCTs activity is reported to be correlated

with the sensitivity of tyrosine kinase inhibitors (TKIs) like imatinib [18]. Furthermore, OCTs determines the responsiveness of platinum derivatives in chemotherapy [19]. OAT2 is the predominant OATs expressed in liver [20]. Functionally, OAT2 works as an ion exchanger that transport endogenous compound including PGE2, cAMP, cGMP and urate. Other exogenous compound including some antibiotics, antivirals, NSAIDs are also transported by OAT2. Corresponding with uptake transporters, efflux transporters existing in apical side of hepatocytes help transport bile acids, other endogenous compounds like bilirubin and xenobiotics into bile.

Bile salt export pump (BSEP) works as the major transporter responsible for the secretion of bile acids from hepatocyte to bile canaliculus. Both conjugated and unconjugated bile acids are transported via BSEP. Limited number of drugs are also transported by BSEP like pravastatin [21]

Multidrug resistant-associate proteins (MRPs) play a significant role in the excretion process. The most abundant MRP expressed in apical side of hepatocyte is MRP2. It is mainly expressed in liver canaliculi and responsible for the secretion of lots of important endogenous compounds including bile acids and estradiol-17-glucuronide into bile. Exogenous compounds including BSP, anticancer drugs and carcinogens. Thus MRP2 plays an important role in the detoxification of exogenous toxins in liver [22].

P-glycoprotein (P-gp, also known as MDR, multidrug resistance protein) was the first recognized efflux transporters as having significant impact on drug distribution. Not only in liver, is it also expressed in intestine, blood-brain and placental barrier and kidney. The expression level of P-gp in intestine is actually higher than that in liver. It

has a wide range of substrates including cationic drugs, steroid hormones, peptides and lipids [23]. The function of P-gp overlaps with MRP2 to some degree. The difference is P-gp normally doesn't transport conjugated compound like glutathione conjugates and sulfate conjugates[24].

Breast cancer resistance protein (BCRP) is expressed in many biological interface including blood-brain barrier, blood-placental barrier and intestine. In the liver, BCRP is also expressed in the canalicular membrane of hepatocytes. It is believed that BCRP is related to a protection mechanism of physiological barriers. Given its specificity to anticancer drugs and environmental carcinogens, BCRP is associated with multidrug resistance in tumors[24].

Multidrug and toxic compound extrusion (MATE) proteins are first recognized as bacterial drug transporters. Back to 2005, two human MATE transporters (MATE 1 and MATE 2) were identified [25]. Genetically, MATE belongs to solute carrier family (SLC) while other efflux transporters belong to ATP-binding cassette (ABC). MATE1 is predominately expressed in the canalicular membrane of hepatocytes. It mediates the efflux of organic cations including creatinine, guanidine and thamine, as well as drugs like metformin and cimetidine.

Few efflux transporters were expressed on the basolateral side of hepatocyte, including MRP3, 4, 5 and OSTα/β. The understanding of these transporters is still limited. It is believed that MRP 3, 4 work as a protective system which compensates the malfunction of efflux transporters on the apical side, though the Mrp3 knockout mice didn't show an expected change in blood bile acids level[26]. MRP 4 seems to

protect the hepatocyte by transporting bile acids in cholestatic condition [27]. The role of MRP5 is still remained unknown. OSTα/β are responsible for the uptake of steroid hormones and bile acids. Different from MRPs, they are an ATP-independent, bidirectional transporters [28]. Corresponding with hepatic uptake transporters, efflux transporters in basolateral sides could mediate a process called “hepatic hopping”, substrates of both uptake transporter and efflux transporters may shuttle in adjacent hepatocytes and distribute into neighboring hepatocytes [29].

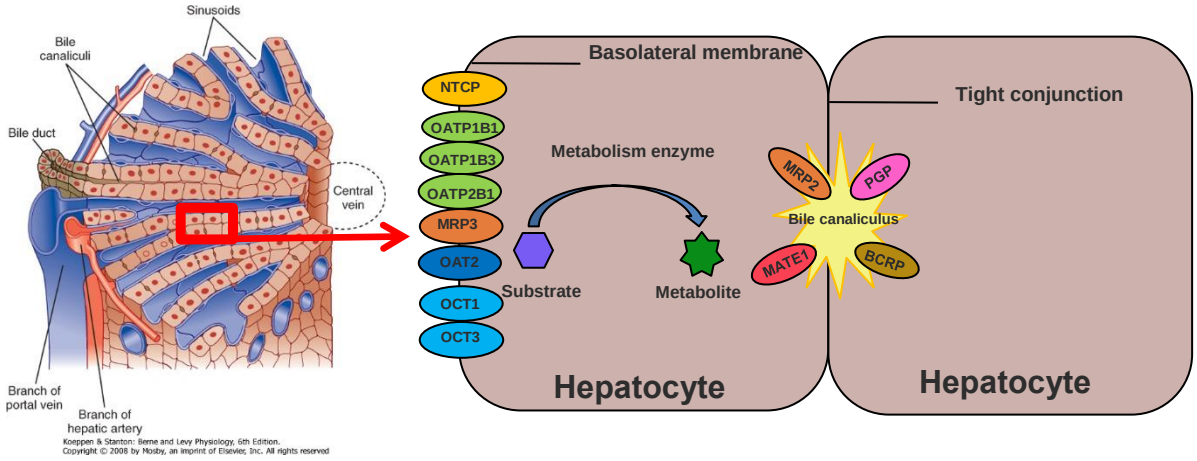

Fig. 1 The anatomical structure of liver and the uptake/efflux transporters expressed in hepatocyte.

Hepatic metabolism serves as the “driving force” of the enterohepatic circulation. Thus, the generation of these metabolites is the rate-limiting step in the circulation of these compounds. Except for few compounds partially excreted into bile as their original forms like pravastatin [30], a compound could not get recycled without metabolism. Except for liver, other organs especially small intestine are also [31] capable of metabolizing compounds. These metabolites generated before reaching

liver are believed to enter systemic circulation and get eliminated by urine since they have already lost their pharmacological activity and toxicity after metabolism. Therefore, liver is believed to be the major source of metabolites generation. That sets the predominant role of the hepatic metabolism in the EHR process.

The pharmacokinetic of a drug could be greatly influenced for its EHR process. Due to the increased chances of getting re-absorbed in distal small intestine, the in vivo exposure of drug will increase. It further results to an increased AUC and bioavailability and indicated a double, even triple peaks in the blood concentration VS time curve. The terminal elimination half-life is increased as well. Thus, the enterohepatic recycling becomes a significant drug disposition process.

The involvement of such metabolism enzymes and transporters also generate the increased possibility of drug toxicity. The down regulation of hepatic metabolism enzymes directly decreases the drug in vivo exposure. However, the decrease of hepatic transporter expression level also reduces the drug in vivo exposure by blocking the enterohepatic recycling. Therefore, the down regulation of transporters increases the risk of drug toxicity in pathologic conditions. Cholestasis is a disease causing decrease in bile flow due to the impaired secretion of hepatocytes or obstruction of bile ducts. The decreased bile flow causes the accumulation of toxins in liver, which result to the liver damage. Thereby the hepatic enzyme expression level and transporter level are dysregulated. The down regulation of efflux transporters causes the obstacle to EHR and leads to the accumulation of metabolites in liver. The accumulation of metabolites leads to substrate inhibition and therefore decrease the aglycone metabolism. The drug in vivo exposure are

increased as a result. Other diseases including viral hepatitis infection, non-alcoholic fatty liver, primary biliary cholangitis and liver carcinoma are reported to cause the decrease of transporters and metabolism enzymes.

Drug mediated inhibition of metabolism enzymes or transporters will also lead to the increased drug exposure. Commonly used anti-HIV drug indinavir is reported to inhibit UGT1A1, 1A3 and 1A7. With the inhibition of UGTs, other drugs metabolized by these UGT enzymes like sorafenib [32], raloxifene [33] and irinotecan [34] will have higher risk of drug toxicity due to the increased drug in vivo exposure. Drugs that are substrate of transporters also have potential of toxicity by inhibiting the EHR of other drugs that sharing the same transporters. Macrolide antibiotics clarithromycin and erythromycin were demonstrated to increase cholesterol lowering drug pravastatin's blood concentration. The increased blood concentration was caused by the inhibition of P-gp and OATP1B1[35].

As a comprehensive drug disposition process, enterohepatic recycling could influence drug in vivo exposure by providing multiple chances for drugs to be re-absorbed before they are eliminated from bile or feces. The metabolism step is the critical step in the EHR process. For most drugs, their metaboilites are the substrate of efflux transporters instead of the aglycones and the biotransformation is required for them to be excreted into bile. The interruption of the EHR by diseases or drugs will cause the increase of drug in vivo exposure and lead to potential drug toxicity. Except for inhibition or down regulation on metabolism enzymes which directly decreases the drug biotransformation, the negative impact on transporters will also increase the drug in vivo exposure.

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Chapter 2 Statement of Problems

Phase II metabolism is the most important step in the enterohepatic recycling. Since the study of EHR came from the study of bile acids recycling, it is assumed that the formation and disposition of phase II metabolites were the same as bile acids.

Bile acids are synthesized by hepatocytes. After they return to the small intestine through bile secretion, most of the bile acids are re-absorbed in small intestine since only 5% of bile acids in the bile are generated de novo [1]. In other words, the bile acids excreted into the small intestine are all originally generated in hepatocytes and subjected to recycling. Therefore, liver serves as a metabolite-forming organ and small intestine as the recycling organ. Bile acids circulating between intestine and liver is termed as “Enterohepatic Recirculation.”

The recycling of drugs is slightly different. Drug metabolites returned small intestine with bile secretion could not be directly reabsorbed in small intestine. They require the participation of gut flora in terminal illume and colon. After hydrolysis by gut flora and converted to their parent form, these drugs could restore their pharmacological activity and re-absorbed in small intestine. Thus, these drugs are recovered from bile and “recycled” between small intestine and liver.

Interestingly, where the phase II conjugates in the bile come from was not specified in textbooks, and it is assumed that they come from the metabolites generated in liver the same as bile acids [2-6] .

However, other organs such as small intestine and liver are also capable of metabolizing drugs into phase II conjugates. These extrahepatic metabolisms are not considered to contribute to EHR as much as hepatic metabolism. One reason is that extrahepatic metabolism has relative lower metabolism rate and capability compared to hepatic metabolism. Another reason is that extrahepatically generated metabolites are considered entering systemic circulation and directly eliminated through kidney, for they likely already lost their pharmacological activities except for a few active phase II conjugates.

In our recent study, we found that some extrahepatically formed flavonoid glucuronides could participate in enterohepatic recycling efficiently instead of being eliminated through kidney. Our previous data indicated that over 50% of the flavonoid glucuronides were recovered in bile after being directly infused to liver. The AUC of these glucuronides in blood (collected from tail vein) and bile was calculated. We found that the AUC of these flavonoid glucuronides in bile was 100 times higher than in blood.

Thus, there are questions remained to be answered.1) could extrahepatic metabolism become another source of phase II conjugates formation? 2) are aglycones better source of biliary glucuronides than their corresponding metabolites? 3) how these metabolites taken up compared to their aglycones?

To answer these questions, we proposed to study the mechanism of the metabolites recycling. We focused on the recycling of glucuronides among all the phase II metabolites for glucuronidation serves as the most important phase II metabolism

path way (45%-70% of xenobiotics are metabolized by glucuronidation) [9]. Considering the hydrophilicity of these glucuronides, it is hard for them to cross the cell membrane by passive diffusion. Therefore, the uptake of these metabolites into hepatocytes requires the participation of uptake transporters. Since OATPs have the widest substrate list and OATPs are the most abundant uptake transporters expressed in the basolateral side of hepatocyte [10], we hypothesize that OATPs play a major role in the recycle of these metabolites. Thus, our central hypothesis is that the OATPs mediated hepatic uptake is the rate-limiting step in the EHR of these glucuronides.

The innovation of this study is that we are the first to focus on the recylcling of the glucuronides, the contribution of extrahepatic metabolism and the mechanisms by which these glucuronides taken up during recycling.

The study of enterohepatic recycling of these metabolites are of clinical significance. The clinical dose management should be adjusted according to the pathological condition that influence drug metabolism and elimination. If liver disease that influence hepatic metabolism is the only factor that taken into consideration, the dose management will be biased due to the underestimated impact of extrahepatic metabolism. Furthermore, some drug metabolites like ezetimibe glucuronide have nearly the same pharmacological activity as their parent form [7.8]. The EHR of these active metabolites will be important in the evaluation of in vivo efficacy for dose management.

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Chapter 3 General Methods

In order to study the mechanism of how some flavonoid glucuronides participate into the EHR and the role of OATPs in the uptake of these glucuronides, we have successfully established a few methods.

1. Bio-synthesize of flavonoid glucuronides.

Most of the flavonoid glucuronides are commercially available. For some of the commercially unavailable glucuronides, a HELA UGT1A9 over expressed-cell line was used for bio-synthesis of the required glucuronides. Briefly, cells were seeded on a 75ml cell culture flask. The flavonoid was prepared in HBSS buffer (pH=7.4) at 20uM concentration working solution. Cell culture medium was removed and cell was rinsed with 10ml warm (37°C) HBSS two times. Then, 20ml of warm (37°C) working solution was added into cell culture flask and incubated with cell for over-night. After incubation, working solution was collected and centrifuged at 4000 rpm for 20min. The supernatant was collected and mixed with 40ml of dichloromethane to extract the un-transformed aglycone. The water phase containing glucuronide was collected after liquid-liquid extraction. The concentration of glucuronide was quantified by UPLC/UV detector [1].

Table 1 Flavonoid glucuronides used in the study

Compound name	Source
Genestein-7-glucuronide	Bio-synthesiz

Chrysin-7-glucuronide	ed
Biochamin A-7-glucuronide	
Wogonin-7-glucuronide(wogonoside)	
Apigenin-7-glucuronide	
Icaritin-3-glucuronide	
Icaritin-7-glucuronide	
Luteolin-3'-glucuronide	Purchased
Baicalein-7-glucuronide(baicalin)	
Quercetin-3-glucuronide	
Scutellarein-7-glucuronide	
Luteolin-7-glycoside	

2. Hepatic portal vein infusion

To test how much glucuronides could be excreted into bile after reaching hepatocytes, a hepatic portal vein infusion study was conducted. Rats (Wistar rats, Male, body weight 280-330g) were fasted for 16 hours before experiment. Rats were anesthetized by i.p. injection of 50% urethane solution (around 1ml/rat). After anesthesia, the rats peritoneal was cut and open. Bile duct was located, separated from adjacent tissues and catheterized with PE-10 tube. The hepatic portal vein was located and catheterized with retention needle. Glucuronides were prepared at required concentration in HBSS buffer (pH=7.4) as working solution. Working solution was infused through portal vein catheterization at the rate of 2 ml/hr. The infusion

lasted for 2.5 hours. Bile samples and blood from tail vein were collected every 0.5 hour. Bile samples were collected to determine the amount of glucuronide secreted into bile. Blood samples were collected to determine the concentration of glucuronide entered systemic circulation.

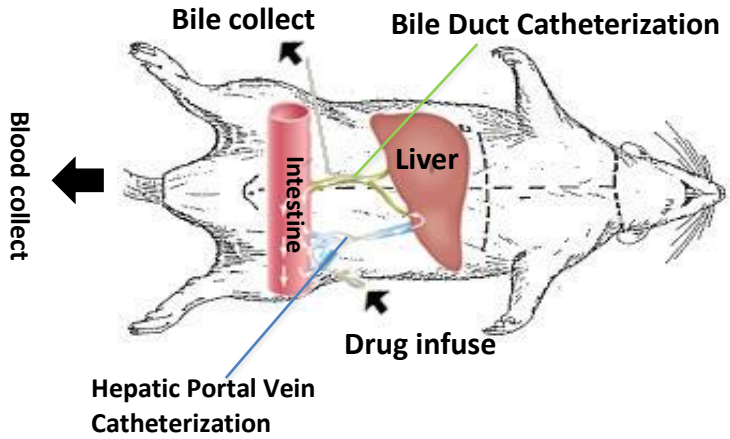

Fig.1 The hepatic infusion model. Drugs infused from hepatic portal vein at the rate of 2ml/hr. Bile and tail vein blood samples were collected. The infusion lasted for 2.5 hours.

3. Small intestine perfusion

To test the impact of intestinal metabolism on the recycling of polyphenol compounds, we conducted a small intestine perfusion experiment. Rats (Wistar rats, Male, body weight 280-330g) were fasted for 16 hours before experiment. Rats were anesthetized by i.p. injection of 50% urethane solution (around 1ml/rat). After anesthesia, the rats peritoneal was cut and open. Small intestine was catheterized at the beginning of duodenum segment and 15cm down-stream, for this segment is the most active absorption part in small intestine. The catheterized segment was flushed with warm HBSS (37°C) before the beginning of perfusion. Flavonoids were prepared

at required concentration in HBSS buffer (pH=7.4) as working solution. Pre-warmed working solution (37°C) was perfused from duodenum catheterization at the rate of 0.193 ml/min (based on our previous experience, small intestine will have plenty of time to absorb given compound at this perfusion rate). Bile duct was located, separated from adjacent tissues and catheterized with PE-10 tube. The perfusion lasted for 3 hours. Perfusate, bile and blood from tail vein were collected every 0.5 hour. Blood from hepatic portal vein was collected at 2.5 and 3 hour time points through hepatic portal vein catheterization. Perfusate was collected to calculate the ratio of the absorbed polyphenol parent compound. Bile was collected to determine the amount of glucuronide (after metabolism) and untransformed aglycones secreted into bile. Blood from tail vein was collected to determine the concentrations of glucuronide and aglycone entered systemic circulation. Blood from portal vein was collected to determine the concentration of glucuronide that was metabolized after intestinal absorption.

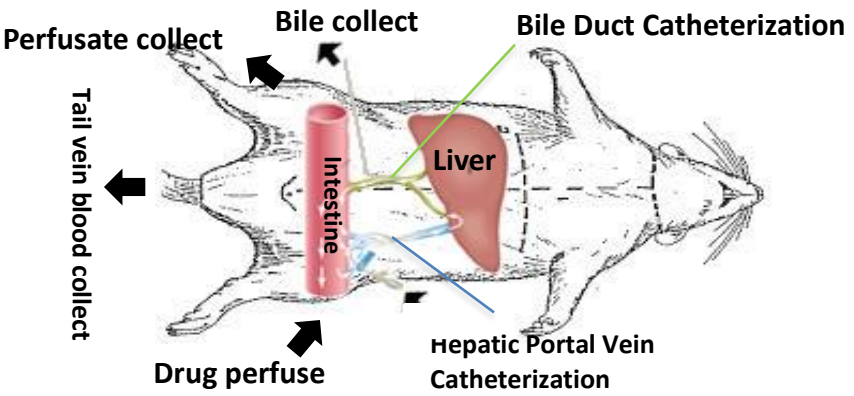

Fig.2 The small intestine perfusion model. Drugs infused from small intestine at the rate of 0.2ml/min. Bile and tail vein blood samples were collected. The infusion

lasted for 2.5 hours. Portal vein blood was collected from portal vein catheterization at the end of the perfusion experiment.

4. Cellular Uptake

In order to test whether these glucuronides are substrate of OATPs, an OATP over-expressed cell uptake study was conducted. Three OATP over-expressed cell lines (HEK 293 OATP 1B1/1B3/2B1 over-expressed cell line) were used in the uptake study. Cell was seeded into 24 well plate 3 days before experiment date. Selected flavonoid glucuronide was diluted in HBSS buffer as working solution. Before incubation, cell culture medium was removed and cell was washed with 400μl 37°C HBSS buffer twice. Working solution was pre-heated to 37°C and 400μl of working solution was added into each well. After incubation, cell was washed with 400μl ice-cold HBSS buffer twice and cell pellet was flushed out by 200μl of HBSS buffer. The cell pellet was further sonicated for 30 min to break the cell and release the intracellular compounds. 150μl of cell pellet was collected and 150μl of acetonitrile (contain 0.2μM rutin as internal standard) was added into the pellet suspension. The suspension was centrifuged at 15,000 rpm for 15min and supernatant was collected for analysis. Intracellular concentration was measured to determine the uptake capability of OATP isoforms to individual glucuronide.

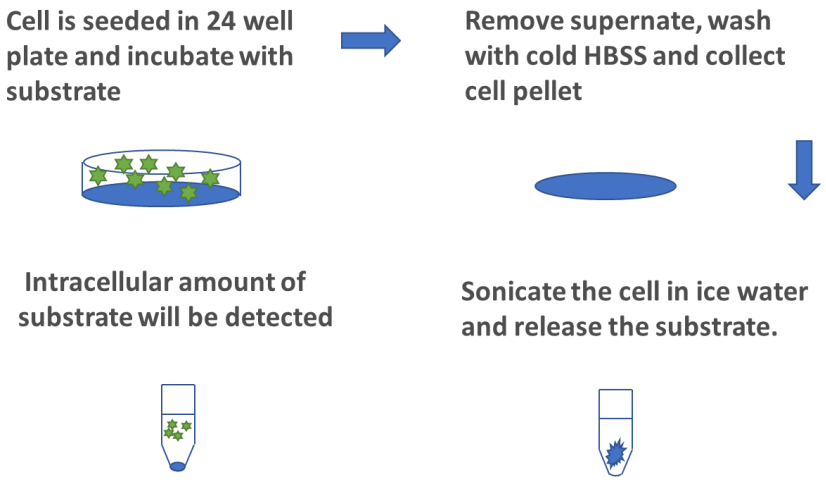

Fig.3 cell uptake experiment was conducted as described. Metabolites were incubated with cells for 20min. Then, cells were collected and processed. Intracellular concentration of metabolites were determined after that.

5. Pharmacokinetic Study

The impact of EHR on glucuronides' in vivo exposure was determined by conducting a single dose pharmacokinetic study. Wogonin/baicalein and ezetimibe/acetaminophen pairs were selected as model compounds for they represented flavonoid and drugs with high and low recycling ratios. Compound pair was prepared in oral suspension at the concentration of 10mg/ml for each compound. Rats (Wistar rats, Male, body weight 280-330g) were fasted for 16 hours before experiment. 30 mg/kg of compounds were dosed to the rat by oral gavage. Blood samples were collected from tail at 0, 0.5, 1, 2, 4, 6, 8, 24 hours after dosing. 4 rats were set in one group to avoid bias. Blood samples were further analyzed by LC/MS method after sample processing.

6. Sample Processing for Analysis

6.1 Blood Sample

10μl of blood was mixed with 10μl of water and 200μl of ACN (with 0.2μM rutin as internal standard) was added into the mixture. After vortex for 1min, the mixture was centrifuged at 15,000 rpm for 15min. The supernatant was dried under nitrogen blowing at room temperature and reconstitute with 100μl of 20% ACN for analysis.

6.2 Bile Sample

5μl of bile was mixed with 5μl of water and further dilute into 1ml of mixture (containing 0.1μM rutin as internal standard). The mixture was further loaded on an Oasis® HLB SPE cartridge and eluted by 1 ml of water, 3ml of 40% methanol. 1 ml of methanol was used as final elution fluid and sample was air-dried after elution. The residue was reconstituted with 200μl 20% acetonitrile for analysis.

6.3 Perfusate Sample

50μl of perfusate sample was mixed with 50μl of water and 100μl of acetonitrile was (with 0.2μM rutin as internal standard) added into the mixture. After vortex for 1min, the mixture was centrifuged at 15,000 rpm for 15min. 100μl of supernatant was taken for analysis.

7. Data Analysis

The bile amount of one compound was calculated by the bile concentration in 30min times the bile secretion amount in 30min. Accumulated bile amount was calculated by the addition of bile amount in the infusion/perfusion lasting time period. Bile secretion

rate was calculated by the linear regression of time versus accumulated bile amount. The slope of the regression line is considered as the bile secretion rate.

Recycle ratio of one compound was calculated as below:

$$\text{RR (Recycle Ratio)}\% = \frac{\text{Biliary secretion rate at steady state}}{\text{Infusion rate to liver/small intestine}}$$

PK profiles were calculated by WinNonlin. Elimination half-life was calculated by using two-compartment model. Elimination half-life was calculated to determine the drug in vivo retention time.

Statistical significance was calculated by using Student *t* test (for n=2) or one-way ANOVA with Turkey post-hoc (n>3). $P<0.05$ (*) was considered having significant difference.

Reference

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Chapter 4 Development and validation of an LC-MS/MS method for the quantification of flavonoid glucuronides (wogonoside, baicalin, and apigenin-glucuronide) in the bile and blood samples: application to a portal vein infusion study.

1 Introduction

Flavonoids, which consists of two phenyl rings and a heterocyclic ring in the structures, are widely distributed in many fruits and vegetables [42]. For example, apigenin, a 5,7,4'-trihydroxyl-flavonoid, is found as one of the major ingredients in chamomile tea and the contents of apigenin is up to 0.8% to 1.2% in a typical chamomile tea drink [43]. Other examples include genistein in soy, quercetin in oranges, anthocyanidins in blueberries [44, 45]. Flavonoids possess multiple pharmacological effects, including anti-cancer, anti-inflammation, and anti-oxidation [46-48], probably due to their anti-oxidation property from hydroxyls. Therefore, this class of compounds is believed to be the active components in nutritional and herbal materials. For example, wogonin was reported as the key active components against inflammation in *Scutellaria Radix* [49].

Although bio-activities of flavonoids are promising, their oral bioavailabilities are usually poor [50, 51], which hampers flavonoids being developed as therapeutic drugs. Pharmaceutical research has demonstrated that one of the major reasons for poor oral bioavailability for flavonoids is glucuronidation in the intestine and the liver [52, 53]. Interestingly, some flavonoid-glucuronides are good substrates of certain

efflux transporters (e.g., BCRP, MRP2) [54] and can be secreted through bile into the intestine, where glucuronides can be hydrolyzed by microflora to release the aglycones, followed by re-absorption in the intestine to form a enterohepatic recycling (EHR) [52, 53]. Many studies have been conducted to study enterohepatic recycling in order to have a better understanding and utilization of this physiological phenomenon. Thus, sensitive and robust analytical method to quantify flavonoids and their glucuronides in the bile and blood samples are required.

A few LC-MS methods for flavonoid-glucuronides quantification in the bile and blood have been published previously [55-60]. However, these methods are not widely cited probably because of low sensitivity and complex sample preparation procedures. Further studies suggested that low signal sensitivity in the bile might be caused by ion suppression from bile acids [61]. In addition, bile acids can form micelles and encapsulate the analytes, resulting in low extraction recovery [62, 63]. Alternatively, a common approach to analyze glucuronides in the bile is to hydrolyze the conjugates and quantify the aglycones [64, 65]. However, this indirect quantification method is not robust and may not be accurate because stability is a concern for some flavonoids during hydrolysis. Moreover, this indirect quantification can't quantify both glucuronides and aglycones at the same time, which is required in many studies.

This chapter developed and validated a LC-MS method for glucuronides quantification in the bile and blood samples and apply the method in a portal vein infusion study, where both bile and blood samples are collected.

2 Experiments

2.1 Chemicals and reagents

Wogonoside was purchased from Meilunebio (Dalian, China). Apigenin-7-O-glucuronide was purchased from HWI Analytik GmbH (Rheinzaberner, Rülzheim, German). Rutin (internal standard, I.S.) was purchased from Cayman Chemistry Company. Baicalin was purchased from Indofine (NJ, USA). DMSO was purchased from Sigma-Aldrich. Acetonitrile and methanol were from Omni Solv (CA, US). HLB cartridge and MCX cartridge were purchased from Waters Oasis (MA, USA). X-AW, C18-W and SCX cartridge were purchased from Strata Phenomenex (CA, USA.). Other chemicals were used as received.

2.2 Instrument and conditions:

2.2.1 *UPLC conditions.* UPLC condition: system, Waters Acquity with diode array; column, Restek Ultra BPh (5 μm, 100 mm×2.1 mm); mobile phase, A 0.1% formic acid in water, mobile phase B acetonitrile; gradient, 10% B (0-0.5 min), 10% B-34% B (0.5-1.0 min), 34% B-60% B (1.0-2.5 min), 60% B-95% B (2.5-6.0 min), 95% B-10% B (6.0-7.0 min); flow rate,0.45 ml/min; column temperature,45 ℃; injection volume, 10 μL.

2.2.2 *Mass spectrometry conditions.* The MS analysis was performed on a Sciex 5500 triple quadrupole mass spectrometer (AB Sciex LLC, Framingham, MA) equipped with an ESI source. The detection was conducted using MRM scan type in positive ion mode. The instrument dependent parameters were: ionspray voltage, 5.5 kV; ion source temperature, 400 °C; nebulizer gas (gas 1), nitrogen, 20 psi; turbo gas (gas 2), nitrogen 20 psi; curtain gas, nitrogen 20 psi. Unit mass resolution was set in both mass-resolving quadruples Q1 and Q3. Compound-dependent parameters were listed in

Table 1.

2.3 Preparation of stock solution, calibration curves, and QC samples in the bile and blood. Stock solutions (1.0 mM) of wogonoside, baicalin, apigenin-7-O-glucuronide, and rutin were prepared in water:acetonitrile = 1:1 (containing 0.1% formic acid). The working solution for bile samples preparation were prepared by serial dilution of the stock solution into 50% methanol to afford 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39 μM, respectively. To prepare calibration curve samples in the bile, these working solutions (5 uL each) were spiked into 5 uL of blank bile, after which the mixtures were further diluted in 1 mL of water (containing 0.1μM rutin as internal standard) and loaded to SPE columns. After eluted from the SPE column with elution solutions described below, samples were collected, and solvents were removed under N_2 flow. The residues were reconstituted into 20% acetonitrile (200 μL) for LC-MS analysis to reach a final concentration of 5,000, 2,500, 1,250, 625, 312.5, 156.3, 78, 39, 20, 10nM. The quality control (QC) samples for each analyte were prepared at three different concentrations (10.0 nM as low, 156.25 nM as medium, and 2,500.0 nM as high) following the same procedures as described above.

The working solution for blood samples preparation were prepared by serial dilution of the stock solution into 50% methanol to afford 4,000, 2,000, 1,000, 500, 250, 125, 62.5, 31.2, 15.6 nM, respectively. To prepare standard curve in the blood, blank blood (10 μL) was mixed with 10 μL of standard curve solutions. Then, the mixture was further added with 200 μL of acetonitrile (containing 100 nM rutin as internal

standard). The mixture was further vortex for 1min and centrifuged at 15,000 rpm for 15min. Then, 200μL of supernatant was collected and dried under nitrogen flow. The residues were reconstituted into 20% acetonitrile (200 μL) to reach a final concentration of 4,00, 2,00, 1,00, 50, 25, 12.5, 6.25, 3.12, 1.56nM for LC-MS analysis.The quality control (QC) samples for each analyte were prepared at three different concentrations (1.56 nM as low, 25.0 nM as medium, and 200.0 nM as high) following the same procedures as described above.

2.4 Sample preparation. Bile samples preparation. The bile samples were prepared following the procedures described in the calibration curve samples preparation. Briefly, 5 uL of bile samples was spiked into 5 uL of 50% acetonitrile, which was further diluted into 1 mL of water for SPE extraction.

Blood samples preparation. Blood samples were also prepared following the procedures described in the calibration curve samples preparation. Briefly, 10 uL of blood samples was spiked into 10 uL of 50% acetonitrile, which was further precipitated by 200 μL of Methanol (with 0.1% formic acid and IS).

2.5 Liquid-Liquid Extraction (LLE) and Solid Phase Extraction (SPE) of bile samples: To establish a reliable extraction method, wogonoside was selected as model compound and T-CA, the most abundant bile acid in rat bile [66], was selected as the marker of bile acid. Methanol, which is a typical solvent used for protein precipitation [67-69], was used in LLE. Briefly, 5 μL of QC samples at three concentrations (low, medium and high) were spiked with 5 μL of blank bile. Then, 1 mL of methanol (with

0.1% formic acid and IS) was applied to precipitate the bile acids and proteins. After vortex and sonicate for 15min, samples were further centrifuged at 15,000 rpm for 15min, after which 950 µL of supernatant was taken and dried under nitrogen flow. Then, the residue was reconstituted into 200 µL of 20% acetonitrile for LC-MS analysis.

To establish an appropriate SPE procedure, five commonly used SPE cartridges were tested. Briefly, wogonoside (5 µL) was mixed with blank bile (5 µL) and diluted to 1 mL of water with 0.1% formic acid at the concentration of 1 µM. Internal standard (rutin) was added in and diluted at 0.5 µM in the mixture. Then, samples were loaded onto the cartridges, which were eluted sequentially with 1 mL of water with 0.1%formic acid (loading fraction) and then 1 mL of methanol with 0.1% formic acid twice (MeOH elution). The collected fractions were dried under nitrogen flow and reconstituted with 200 µL of 20% acetonitrile for LC-MS analysis.

For elution optimization, different percentage of methanol applied in elution solutions were tested. Briefly, after sample loading, 30%, 40%, 50%, or 60% of methanol (1.0 mL) were eluted respectively to afford the pre-elute fractions (Fr. 1). Then, 100 % of MeOH was used to eluted to afford MeOH fraction (Fr. 2). After that, solvents were dried under nitrogen blowing and the residues were re-constituted into 20% of acetonitrile (200 µL) for LC-MS analysis.

2.6 Method validation

The analytical method was validated according to the “Guidance for Industry,

Bioanalytical Method Validation" presented by the US Food and Drug Administration (2018)[70].

2.6.1 Specificity. The specificity of the method was determined by injecting processed pooled blank bile and pooled blank blood samples.

2.6.2 Linearity and lowest limit of quantification (LLOQ): Calibration curves were prepared as described before. After sample analysis, a least-square linear regression ($1/X^2$ weights) was applied to determine the slope, intercept and correlation coefficient factor. The lowest limit of quantification (LLOQ) was determined accurately and precisely with a signal-noise ratio (S/N) of at least 5:1.

2.6.3 Recovery, matrix effect, and carry-over: The recovery was evaluated by comparing the peak areas obtained from samples prepared from blank bile/blood spiked with QC samples with those from samples prepared from water spiked with the same concentrations.

The matrix effect was determined by comparing the peak areas of samples prepared from residues of pooled bile/blood spiked with QC samples with those from residues of water spiked with the same concentration of QC samples.

The carryover impact was evaluated by inject blank matrix (50% acetonitrile, no analyte and IS.) right after the injection of the highest concentration of calibration standards.

2.6.4 Effect of T-CA on wogonoside's ionization: The effect of T-CA on wogonoside's ionization was evaluated by comparing the peak areas of QC samples at a low (156.2 nM) and a high concentration (2,500 nM) spiked with T-CA (20 mg/mL) with those spiked with water.

2.6.5 Precision and accuracy. Precision and accuracy were conducted by replicate the analysis of QC samples (n=3). The intra-day accuracy was conducted by performing the analysis in same day and 3-day accuracy was conducted by performing the analysis in consecutive 3 days.

2.7 Application to a portal vein infusion experiment

Animals. The animal protocol used in this study was approved by University of Houston Institution of Animal Care and Use Committee. Male Wistar rats (270~320 g) were housed in an environmentally controlled room (temperature: 25 ± 2℃, humidity: 50% ± 5%,12h day-night time cycle) at least a week before the experiment.

Portal vein infusion: The portal vein infusion study has been performed using a method was previously published by us [71]. Briefly, rats were fasted over-night with free access of water before the day of experiment. After a rat was anesthetized, its superior mesenteric vein in the mesentery of jejunum, which is directly connected to the portal vein, was exposed. The superior mesenteric vein was punctured by a venous indwelling needle. Then, rat's bile duct was located and separated from adjacent tissues. After that, a small cut was made with a micro vascular scissor and a tubing (PE10) was inserted and secured with a surgical suture. Its bile duct was also

cannulated as described [71] . Drug solutions were perfusate from the portal vein cannulation and bile samples were collected from bile duct cannulation. The samples were collected every 0.5 hours.

3 Results

3.1 Method development:

Both UPLC and MS conditions were optimized. For LC condition, formic acid (0.1%) in water and acetonitrile were used as the mobile phases. For MS conditions, positive mode was selected for all analytes based on the method specificity and signal intensity comparing with negative mode. MRM scan type was used to increase the specificity of the method. Each compound was tuned and optimized separately to improve the analysis sensitivity. The parent ions and fragment ions with highest abundance were selected as the ion pairs for each analyte. The compound dependent parameters were summarized in Table 1. Rutin was selected as internal standard (I.S.).

Table.1 compound dependent parameter of the analytes and I.S.

Compound	Q1/Q3	DP	CE	EP	CXP
Wogonoside	461.0/285.0	30	26	10	16
Baicalin	447.0/271.0	31	37	10	17
Apigenin-7-O-glucuronide	447.0/271.0	31	37	10	17
T-CA	514.0/80.0	120	78	10	16
Rutin (I.S.)	611/303	120	47	10	13

3.1 Liquid-liquid extraction (LLE) for bile sample preparation. The results of LLE were summarized in Table 2. The recovery and matrix effect were out of the acceptable ranges (85-115%), especially at low concentration, suggesting components in the bile suppressed ionization of the analytes. Protein precipitation using organic solvent was not an appropriate method for bile sample preparation.

Table 2. The recovery and matrix effect of wogonoside in Liquid-liquid extraction in the bile.

Concentration	Recovery (%)	Matrix effect (%)
Low	ND	ND
Medium	70.6±2.3	61.3±5.2
High	76.7±6.4	69.1±1.3

ND: not detected

3.2 Solid Phase extraction (SPE) for bile sample preparation. First, the retention of wogonoside on the SPE cartridges were tested. The results showed that <15%, which is the acceptable analytical error range, of the loading wogonoside was detected in the loading fraction when HLB cartridge used (Fig 1A) and >85% of wogonoside was in the MeOH elution (Fig 1B). Based on this finding, HLB cartridge was selected as the SPE cartridge.

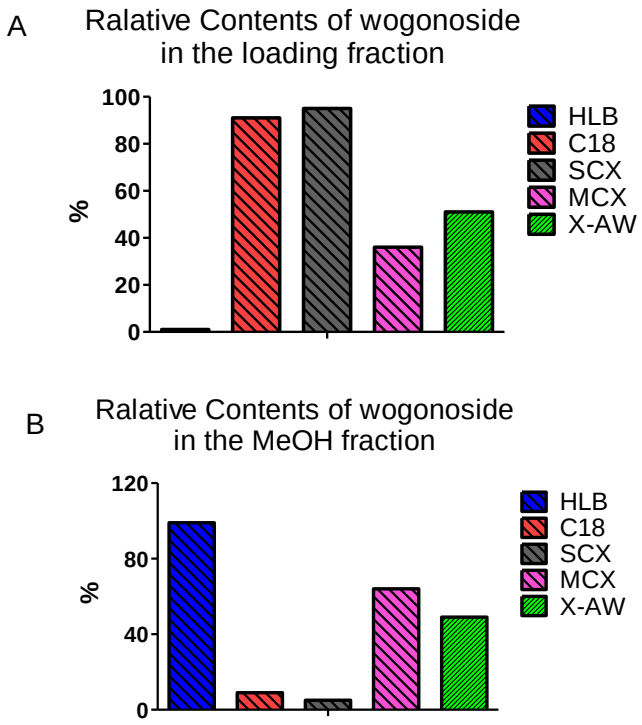

Fig 1 The retention of wogonoside on different SPE cartridges.

Then, the elution condition was optimized based on the wogonoside's recovery and its separation with T-CA in the elution. The results showed that when 40% of MeOH was used as the pre-elution solvent, T-CA can be eluted entirely (>85%, Fr. 1 in Fig 2) and only tiny amount of T-CA (<15%) was in the final elution (Fr. 2 in Fig 2). While for wogonoside, 40% MeOH pre-elution couldn't elute wogonoside (<15%, Fr. 1 in Fig 2) and most of wogonoside (>85%) was in the final elution (Fr. 2, Fig 2). These findings revealed that with 40% of MeOH pre-elution, wogonoside can be separated with T-CA on HLB cartridge. Therefore, of MeOH pre-elution will be used in the preparation of bile samples.

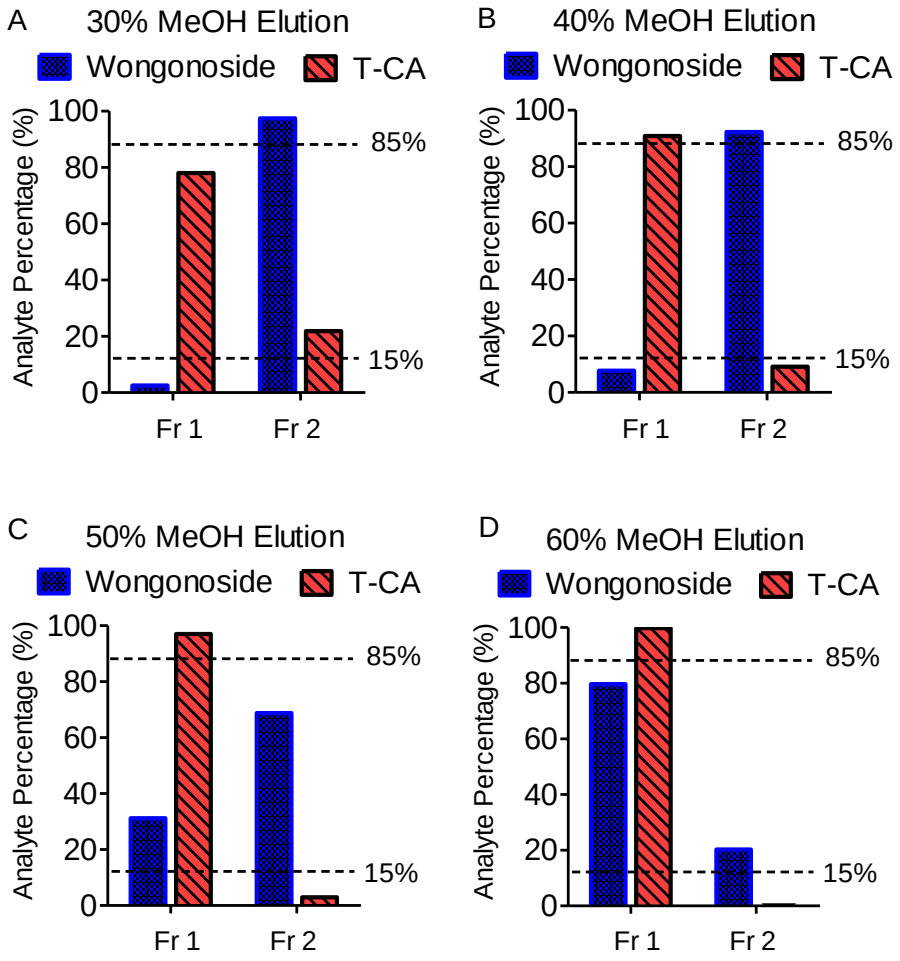

Fig 2 Recovery and separation of wogonoside in SPE extraction with HLB cartridge. (Fr. 1, pre-eluted fraction, Fr. 2 100 % MeOH eluted fraction).

3.4 Effect of T-CA on wogonoside's ionization. The effect of bile acid on wogonoside's ionization was determined by spiking T-CA into QC sample. The spiked T-CA was 20 mg/mL, which is a concentration lower than the real bile samples but close to the solubility limit of T-CA in water.[66, 72]. The results showed that in the presence of T-CA, the intensity of wogonoside was decreased when compared to those spiked with water (Fig 3). This result suggested that components in the bile

(e.g., bile acid) suppress glucuronide's ionization. Therefore, remove bile acids in a key step in sample preparation in LC-MS analysis.

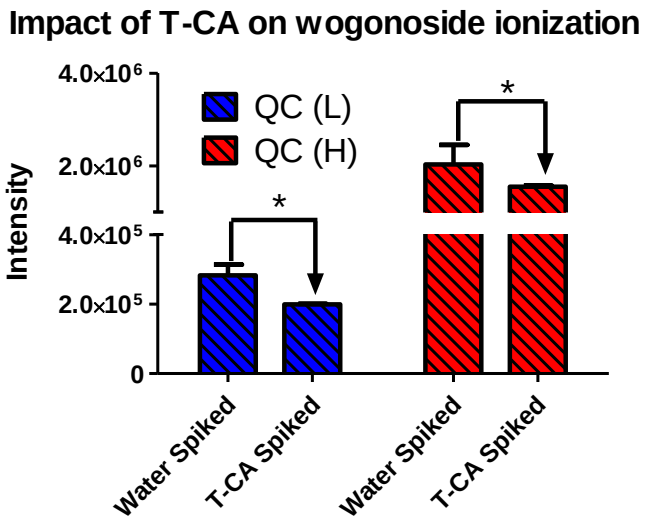

Fig 3. The impact of T-CA on wogonoside ionization.

3.5 Method validation:

3.5.1 Selectivity. The specificity was determined by comparing the samples at LLOQ with those from blank bile and blood samples pooled from six animals. As shown in Fig 2, no significant interfering was observed with the analytes including baicalin, wogonoside and apigenin-7-O-glucuronide.

3.5.2 Linearity and LLOQ. In the bile, the calibration curves were linear from 10.0 nM to 5,000.0 nM for wogonoside, from 10.0 nM to 5,000 nM for baicalin, and from 10.0 nM to 5,000.0 nM for apigenin-7-O-glucuronide. The correlation coefficient (r^2) was at least 0.98 for these glucuronides (Table 3). The lower limit of quantification (LLOQ) for these 3 glucuronides are 10 nM. In the blood, calibration curves were linear from 1.56

nM to 4,000.0 nM for wogonoside, from 1.56 nM to 4,000.0 nM for baicalin, and from 1.56 nM to 4,000.0 nM for apigenin-7-O-glucuronide. The correlation coefficient (r^2) was at least 0.99 for these glucuronides (Table 3). The lower limit of quantification (LLOQ) for these 3 glucuronides are 1.56 nM.

Table 3. The linearity, LLOQ, and R^2 of wogonoside, apigenin-7-O-glucuronide and baicalin in the bile and blood.

Analytes	Linearity		LLOQ (nM)		R^2	
	Bile	Blood	Bile	Blood	Bile	Blood
Wogonoside	10.0-5,000.0	1.56-4,000.0	10.0	1.56	0.983	0.991
Apigenin-7-O-glucuronide	10.0-5,000.0	1.56-4,000.0	10.0	1.56	0.996	0.989
Baicalin	10.0-5,000.0	1.56-4,000.0	10.0	1.56	0.995	0.992

3.5.3 Matrix effect, recovery and carryover effects. In the bile, the extraction recoveries were evaluated using QC samples (n=6) at low (10.0 nM), medium (156.2 nM) and high (2,500 nM) concentrations. The mean recovery was over 95%, suggesting that SPE could extract these glucuronides from bile. The matrix effect after SPE preparations were within the range of 85%-105% for wogonoside, 95%-105% for baicalin, and 95%-105% for apiginen-7-O-glucuronide (Table 4).

In the blood, the matrix effect and recoveries in all three samples were all close to 100%, indicating that the method was good enough to analyze these glucuronides in blood samples without hydrolysis.

To determine carryover, blank matrix samples were injected right after the highest concentration of calibration standards. The results showed that the analytes and IS peaks detected in the blank matrix in both blood and bile are <20% of LLOQ.

Table 4. The recovery and matrix effect of wogonoside, apigenin-7-O-glucuronide and baicalin in bile and blood matrix

Analytes	QC*	Recovery (%)		Matrix effect (%)	
		Bile	Blood	Bile	Blood
Wogonoside	Low	88.3±13.2	99.8±8.1	99.2±12.4	103.1±3.3
	Medium	100.5±15.1	86.7±7.5	98.4±15.7	104.8±8.2
	High	114.4±1.3	87.3±2.4	85.6±3.7	102.1±1.1
Apigenin-7-O-glucuronide	Low	88.2±7.5	88.7±7.2	103.6±10.4	103.1±3.9
	Medium	103.2±5.2	90.7±8.3	101.5±19.1	104.4±8.2
	High	92.5±4.5	92.2±3.3	98.1±11.6	101.3±1.4
Baicalin	Low	103.3±5.3	89.4±7.2	104.4±7.5	103.5±3.3
	Medium	105.2±5.7	98.1±4.7	92.8±4.4	103.6±7.8
	High	94.2±5.2	100.8±3.3	90.4±3.3	101.8±1.2

*QC: Low, medium, and high were 10, 156, 2,500 nM in the bile and 1.56, 25, and 2,000 nM in the blood, respectively.

3.6 Application of the LC-MS method to analyze bile and blood samples derived from a portal vein infusion experiment in rats.

The application of the established method was demonstrated by conducting rat portal vein infusion experiment. Wogonoside, baicalin and apigenin-7-O-glucuronide. The results showed that when the compounds were infused through the portal vein, the analytes can be detected in both the bile and blood (Fig 4), suggesting that these three flavonoid glucuronides were taken up by the hepatocytes and then secreted to the apical side into the bile or basolateral side into the blood. Since glucuronides are highly hydrophilic, uptake transporter may be involved in the liver. Further studies need to focus on transporter identification to fully elucidate the mechanism. The recovery of

wogonoside in the bile was significantly higher than the other two compounds, suggesting that the efficiency of enterohepatic recycling of wogonoside is better than the other two compounds. For baicalin, both biliary and blood exposure were very low, indicating that this compound might have a very fast clearance through urine.

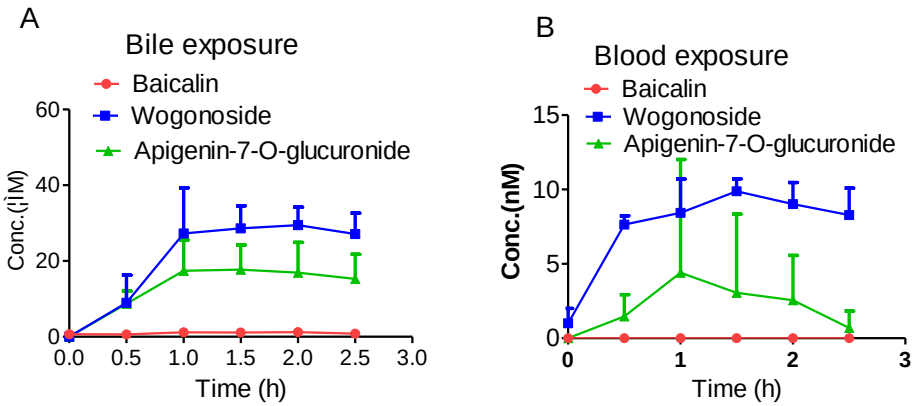

Fig 4 The concentrations of wogonoside, apigenin-7-O-glucuronide, and baicalin in the bile and blood in a portal vein infusion study.

4 Conclusion

In conclusion, a sensitive LC-MS method for directly analyzing flavonoid glucuronides in the bile and blood was established and validated. The method was applied in a portal vein infusion study. The current data suggested that bile acids suppress glucuronides ionization and SPE with HLB can efficiently separate flavonoid-glucuronides from bile acid in bile matrix. More studies are needed to further investigate the mechanism of enterohepatic recycling and clearance of these glucuronides.

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Chapter 5 Hepatoenteric Recycling: A New Disposition Mechanism for Orally Administered Phenolic Drugs and Phytochemicals

1. Introduction

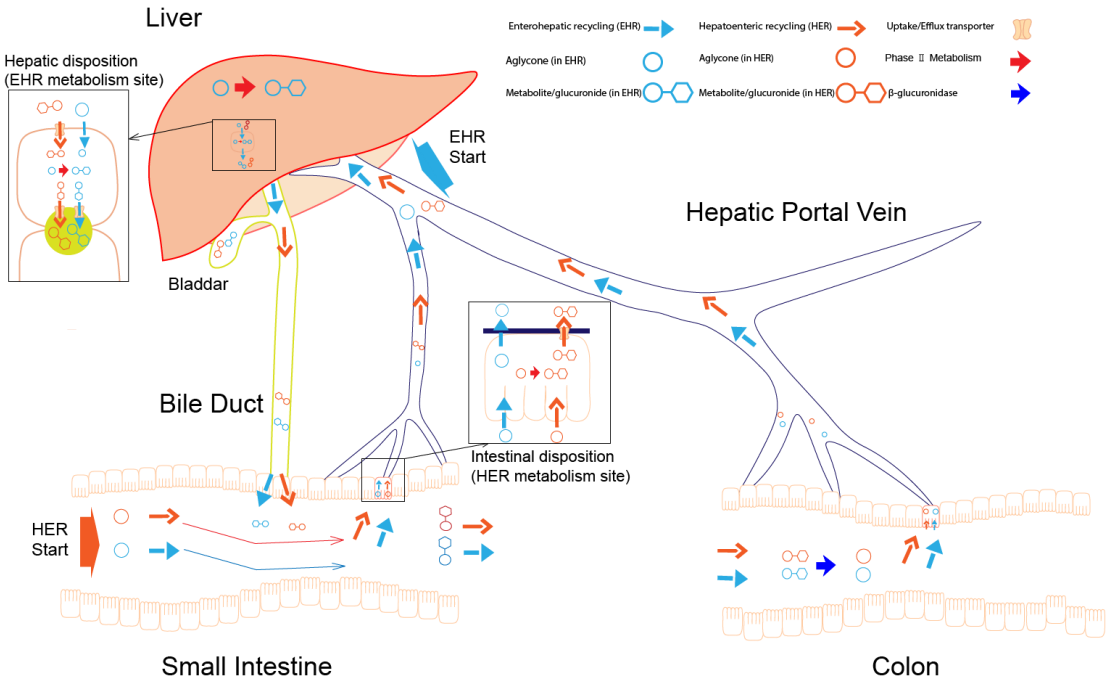
Enterohepatic recirculation/recycling (i.e., EHR) refers to the circulation of biliary acids, bilirubin, drugs or other substances from the liver to the bile, followed by entry into the small intestine, absorption by the enterocyte and transport back to the liver, according to several modern pharmacology textbooks[73]. EHR was first conceptualized in 1950s for bile acid recirculation [74]. In the 1960s, this concept was subsequently used for drugs and xenobiotics that undergo phase II metabolism (a detoxification mechanism) via phase II enzymes like uridine 5'-diphospho-glucuronosyltransferase (UGTs) and sulfotransferases (SULTs), although the word “recirculation” was changed to “recycling” since the aglycone has to be regenerated by microflora [75]. Currently, a variety of drugs including anticancer (e.g., sorafenib, SN-38), anti-cholesterol (e.g., ezetimibe), anti-osteoporosis (e.g., raloxifene), analgesics (e.g., morphine) and others (e.g., mycophenolic acid for organ transplant) are known to undergo EHR [76-80].

The definition of EHR does not define or specify the sources of phase II metabolites in the bile. Traditionally, it is postulated that the glucuronides found in bile are mainly produced in liver, since bile acids are known to be synthesized

in liver. Although bile acid conjugates are absorbed intact from the intestine and then transported into the hepatocytes as conjugates, there is no evidence that glucuronides, as one of the major phase II metabolite, can be efficiently absorbed intact from the intestine. Hence, we typically called this process (involving glucuronides) recycling not recirculation. Moreover, most investigators have held the notion that majority of the drug conjugates found in bile are formed in liver just like bile acid conjugates. Many investigators, including ourselves used to state that liver is the more important organ for EHR than intestine as long as the glucuronidation rate in liver was much faster than intestine in microsome studies [81-88]. Recently, we found that certain phenolic glucuronides are rapidly and efficiently taken up by hepatocytes and then rapidly excreted into the bile, whereas other phenolic glucuronides are not [71]. These phenomena suggest that glucuronides, which need the basolateral hepatic uptake transporters (e.g., OATPs) to gain entry into hepatocytes because of their high hydrophilicity, can be a source of substrates for apical efflux transporters (e.g., MRP2), leading to their excretion into the bile.

Organic anion transporting polypeptides (OATPs) is one class of the major uptake transporters expressed in liver that could contribute to the uptake of phenolic glucuronides. Recently, several reports have shown that polymorphisms in OATPs will influence the pharmacokinetics of drugs and their glucuronides [76, 77, 89, 90], but their role in EHR remains unclear. One study

showed that fecal excretion of ezetimibe was decreased in patients with lower functional activity of OATP1B1 [77], suggestive of its likely role in ezetimibe's EHR. Nevertheless, hepatic uptake of circulatory glucuronides, regardless of its sources, is not recognized as a major force driving EHR [91].



Pic.1 The mechanism of HER and EHR. HER starts at intestine and EHR starts at hepatic portal vein. The differences between these two is the metabolism site (HER in small intestine and EHR in liver). The β-glucuronidase by gut flora is required for the re-absorption of the aglycones because most of the glucuronides could not be directly absorbed in small intestine.

Therefore, the purpose of this systematic study is to provide the first set of evidence that supports a comprehensive redefinition of the EHR so that we

know the sources of glucuronides in the bile during the disposition of phenolic drugs and naturally occurring phytochemicals including flavonoids and polyphenols in vivo. Our study seeks to answer these questions: (1) how efficient are extrahepatically produced glucuronides excreted into the bile; (2) are their corresponding aglycones better sources of biliary glucuronides; and (3) how are these glucuronides taken up? In analyzing the results of our study, a new concept called “Hepatoenteric Recirculation/Recycling (HER)” is proposed to distinguish it from EHR. In HER, intestine is the organ for metabolite formation and liver is the organ for metabolite recirculation. The new HER concept delineates more clearly the complex relationships that describe the disposition of phenolic drugs and naturally occurring phytochemicals such as flavonoids.

MATERIALS AND METHODS.

2.1 Materials and Reagents

Apigenin-7-O-glucuronide was purchased from HWI Analytik GmbH (Rheinzaberner, Rülzheim, German). Wogonin-7-glucuronide, quercetin, scutellarin, luteolin-3'-glucuronide, luteolin-7-glucuronide, wogonin, luteolin, icaritin, icaritin-3-glucuronide and icaritin-7-glucuronide were purchased from Meilunebio (Dalian, China).Estradiol-17β-glucuronide (E2G) and estrone-3-sulfate (E1S) were purchased from Steraloids Company. Raloxifene, raloxifene-4'-glu, raloxifene-6-glu, ezetimibe and ezetimibe phenoxy glucuronide were purchased from Toronto Research Chemical. Apigenin, baicalein and baicalein-7-glucuronide was purchased from Indofine (NJ, USA). Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich. Acetonitrile (ACN) and methanol (MeOH) were purchased from Omni Solv (CA, US).

Chrysin-7-glucuronide, genestein-7-glucuronide and biochamin A-7-glucuronide was generated by our lab via bio-synthesis as described in method 2.2.1.

2.1.1 Animals. Experiments were performed on adult male and female Wistar rats weighing 280-330g (male) or 220-270g (female) at the date of the experiment. The rats were housed in a conventional facility with a 12-hour light/dark cycle. All rats received a standard diet and water. The Rats were fasted for 16 hours before the experiment. All procedures were approved by the

Institutional Animal Care and Use Committee at the University of Houston and were in accordance with National Institutes of Health guidelines for care and use of laboratory animals.

2.1.2 OATP-Overexpressing Cells. OATP 1B1 and OATP 1B3 over-expressed HEK-293 cells, also described in a publication, were kindly shared by Dr. Yue Wei from University of Oklahoma Health Science Center [92]. OATP 2B1 over-expressed HEK-293 cell, first described in a publication, was kindly shared by Dr. Per Artursson's lab from Uppsala University (Uppsala, Sweden) [93].

2.2 Methods.

2.2.1 Bio-synthesis of flavonoid glucuronides. Selected flavonoid glucuronides were bio-synthesized by using Hela-UGT1A9-MRP3 cells developed in our lab [94]. Briefly, the cells was seeded into 75cm^2 cell culture flask and incubated with flavonoids at a concentration of 20uM in 20ml of Hanks' Balanced salt solution (HBSS) buffer. The incubation lasted for 24 hours. After incubation, the cell culture medium was collected and centrifuged at 4000 RPM for 20 min. The supernatant was collected and mix with 40ml of dichloromethane to extract the unreacted aglycone. The water phase containing glucuronide was collected after liquid-liquid extraction. The concentration of glucuronide was quantified by UPLC/UV detector using a previously published method [95]

2.2.2 Hepatic portal vein infusion. This method was the same as described previously and used without modification [96]. Briefly, the hepatic portal vein and bile duct was catheterized after the anesthesia by urethane. The flavonoid glucuronides were infused from portal vein catheterization at the rate of 2 ml/hr. Bile samples were collected from bile catheterization and blood samples were collected from tail by the tail snipping method. The infusion lasted for 2.5 hours and samples were collected every 0.5 hour. The recycle ratio (RR %) was calculated to evaluate the recycling efficiency using the following equation:

Equation 1:

$$\text{RR (Recycle ratio)}\% = \frac{\text{Steady-state biliary glucuronide excretion rate}}{\text{Portal vein infusion rate}}$$

The glucuronide secretion rate at the steady-state was calculated by the linear regression of accumulated amount excreted in bile vs. time curve.

Concentration-dependent wogonin-7-glucuronide infusion experiments. The portal vein infusion experiments were conducted as described previously. Wogonin-7-glucuronide was selected as a model compound. Wogonin-7-glucuronide was prepared at 2, 10, 25, 100 and 1000μM individually and infused into hepatic portal vein.

Aglycones infusion. The experiment was conducted as described earlier. Wogonin, luteolin, apigenin, baicalein, ezetimibe and raloxifene were selected and prepared at 10uM concentration for the infusion experiments.

Aglycone infusion with two different concentrations of wogonin. The infusion study was conducted as described previously with some minor modifications. After 2.5 hours of 2uM wogonin infusion, the infusion solution was switched to 100uM wogonin and infused for another 2 hours. This study was conducte to determine if difference in protein binding of aglycone could have affected their availability to be taken up by liver.

Hepatic infusion of different flavonoid glucuronides. The infusion study was conducted as described earlier. Thirteen glucuronides: wogonin-7-glucuronide, apigenin-7-glucuronide, baicalein-7-glucuronide, luteolin-3'-glucuronide, quercetin-3-glucuronide, scuttelarin (scuttelarein-7-glucuronide), icaratein-3'-glucuronide, icaratein-7-glucuronide, luteolin-7-glucoside, ezetimibe-4'-glucuronide, acetaminophen-glucuronide, raloxifene-4'-glu and raloxifene-6-glu, prepared at 10uM concentration were used in the infusion study. This experiment was used to explore the structure activity relationship.

2.2.3 Cell study

Cellular uptake of flavonoid glucuronides and their aglycones by OATP over-expressed cell lines. Three OATP over-expressed cell lines (HEK 293 OATP 1B1/1B3/2B1 over-expressed cell lines) were used in the uptake study because they are the most important hepatic uptake transporters [97]. Briefly, cell was seeded into 24 well plate 3 days before the experiment. Selected flavonoid glucuronide was diluted in HBSS buffer as working solution. Before incubation,

cell culture medium was removed and cell was washed with 400µl 37°C HBSS buffer twice. Working solution was pre-heated to 37°C and 400µl of working solution was added into each well. After incubation, cell was washed with 400µl ice-cold HBSS buffer twice and cell pellet was collected in 200µl of HBSS buffer. The cell pellet was further sonicated for 30 min and 150µl of cell pellet was collected and 150µl of acetonitrile (contain 0.2µM rutin as internal standard) was added into the pellet suspension. The suspension was centrifuged at 15,000 rpm for 15min and supernatant was collected for analysis.

Time-dependent cell uptake study. The cell uptake study was conducted as described before. Selected flavonoid glucuronides were diluted to 10µM in HBSS as working solution. Working solution was incubated with cell for 0, 10 and 20 min. 10µM flavonoid glucuronide incubated with 50µM OATP inhibitor (rifampicin for OATP 1B1/1B3 and erlotinib or telmisartan for OATP 2B1) for 20min was set as the control to determine the contribution of an OATP transporter.

Concertation-dependent cell uptake experiments. The cell uptake study was conducted as described previously. Selected flavonoid glucuronides were diluted to 0.5, 1, 5, 10, 25 and 50µM as working solutions. Working solutions were incubated with cell for 20 min. The K_m and V_{max} values were calculated by using the Michaelis-Menten equation.

Cross inhibition experiment in OATP1B1 cells using glucuronides and their corresponding aglycones. Several known substrates and non-substrates of OATP1B1 was applied as an inhibitor to inhibit the uptake of 3 selected substrates (wogonin-7-glucuronide, E2G and luteolin-3'-glucuronide). Substrate concentration was 1μM while inhibitor concentration was 25μM. Substrate incubated with cell without inhibitor was set as control group (100% uptake). Relative uptake percentage was calculated by comparing intracellular concentrations of all experiment groups with the control group.

2.2.4 Inhibition study in rat hepatic infusion model.

A mixture of 1mM rifampicin (OATP 1B1/1B3 inhibitor), 1mM telmisartan (OATP 2B1 inhibitor), 1mM E2G (OATP 1B1/1B3 substrate) and 1mM E1S (OATP 2B1 substrate) were prepare in HBSS buffer and used. The experiment conduction was the same as we described earlier with minor modifications. To achieve a better inhibition effect, rats were infused with the inhibitors combination for 1 hour before the beginning of infusion with flavonoid glucuronide. After 1 hour of treatment, selected flavonoid glucuronides were infused with inhibitor combination for 2.5 hours.

2.2.5 Impact of cassette dosing in hepatic infusion model.

The infusion study was conducted as described before. A mixture of 4 glucuronides (wogonin-7-glucuronide, apigenin-7-glucuronide, luteolin-3'-glucuronide and baicalein-7-glucuronide) were prepared at 10 μM together and infused simultaneously.

2.2.6 Comparison of RR% in male versus female rats.

The infusion study was conducted as described before. Wogonoside, baicalin and luteolin-3'-glu were prepared at 10 μM concentration. The study was conducted in female rats.

2.2.7 Small intestine perfusion study.

To investigate the impact of intestinal metabolism on bile accumulation of phase Ⅱ metabolites, a small intestine perfusion study was performed. Briefly, the rat was anesthetized by urethane. Duodenum and 15 cm downstream of small intestine from duodenum was catheterized. 2 μM of flavonoid aglycones (wogonin, apigenin, baicalein) and phenolic drugs (ezetimibe, raloxifene) was prepared in HBSS buffer (pH=7.4) and perfused through intestinal catheterization. The perfusion solution was heated by water bath set at 37°C. The infuse rate was set at 0.2ml/min. The perfusion lasted for 2.5 hours. Bile samples and perfusate were collected. At the last time point, blood samples

from tail vein and from hepatic portal vein were also collected and analyzed. The absorption (%) was calculated as below:

Equation 2:

$$\text{Absorption (\%)} = \frac{\text{Infused amount of aglycone - amount of aglycone in perfusate}}{\text{infused amount of aglycone}}$$

2.2.8 Pharmacokinetic study of wogonin/baicalein and ezetimibe/acetaminophen pairs.

To investigate whether the HER potentials have in vivo impact, a pharmacokinetic experiment was conducted using 4 rats in each group. Briefly, wogonin and baicalein were prepared together in oral suspension at the concentration of 50 mg/ml. Drugs were given to animals at the dose of 30 mg/kg. Ezetimibe/acetaminophen were prepared and dosed the same way as described for the other 2 flavonoids. Blood samples were taken at 0, 0.5, 1, 2, 4, 6, 8 and 24 hours after dosing. Samples were analyzed by LC/MS method after sample process.

2.2.9 LC/MS analysis of blood, bile, cell and perfusate samples.

Blood. A volume of 10μl blood was mixed with 10μl of water and 200μl of ACN (with 0.2μM rutin as internal standard) was added into the mixture. After vortexing for 1min, the mixture was centrifuged at 15,000 rpm for 15min. The

supernatant was dried under air at room temperature and reconstitute with 100μl of 20% acetonitrile for analysis.

Bile. A volume of 5μl bile was mixed with 5μl of water and further dilute into 1ml of mixture (containing 0.1μM rutin as internal standard). The mixture was further loaded on an Oasis® HLB SPE cartridge and eluted by 1 ml of water, 3ml of 40% MeOH. One milliliter of MeOH was used as final elution fluid and sample was dried under air at room temperature after elution. The residue was reconstituted with 200μl 20% ACN for analysis.

Cells. Cell samples were processed by adding 150μl ACN (containing internal standard) into 150μl of cell pellet. After centrifuge at 15,000 rpm for 15min. The supernatant was collected for analysis.

Perfusate. A volume of 50μl perfusate sample was mixed with 50μl of water and 100μl of ACN was (with 0.2μM rutin as internal standard) added into the mixture. After vortex for 1min, the mixture was centrifuged at 15,000 rpm for 15min. 100μl of supernatant was taken for analysis.

2.3 The correlation analysis of recycle ratio and cell uptake results.

The recycle ratios generated from rat infusion experiment were correlated with the intracellular concentrations generated from cell uptake results. Since cell

studies were conducted using human OATPs and human cells, it was important to determine if there is a statistical correlation between these two parameters using the Fisher exact test. To conduct Fisher exact test, all the compounds were classified into 4 groups by their recycle ratio (high and low) and intracellular concentration (high and low). Multiple expression levels of OATP expression was then used to estimate the intracellular concentrations and then correlated with RR% to determine the correlation coefficients (see Supplement method).

We also fitted cellular concentrations determined directly from the experiments and correlated them with RR% to estimate if we can use an Emax model to describe the correlation. The relative expression level close to human liver OATP isoform expression (Supplement table S3) level was applied in the calculation of cellular concentration.

RESULTS

3.1 Portal Vein Infusion Studies

The portal vein infusion studies were performed to measure the difference in recycle ratio (RR%) between different compounds and several aglycone and glucuronide pairs in an effort to map out how significant it is to study this recycling phenomenon we observed previously for 4 flavonoid glucuronides [96].

3.1.1 Concentration-dependent wogonin-7-glucuronide infusion.

The biliary secretion of wogonin-7-glucuronide reached steady state after 1 hr (Fig.1A1,1A2) and remained steady for the duration of the study (ended at 2.5 hr). With increasing wogonin-7-glucuronide infusion concentration, biliary secretion (Fig.1A1) and blood concentration (Fig.1A2) also increased. The biliary secretion of wogonin-7-glucuronide reached a steady state for the secretion amount in 0.5 hrs remained the same after 1 hr infusion. (Fig. 1A1), and that rate was then used to calculate the RR% using Equation 1. The results indicated that RR% was lower at the higher infused wogonin-7-glucuronide concentration (Fig. 1A3).

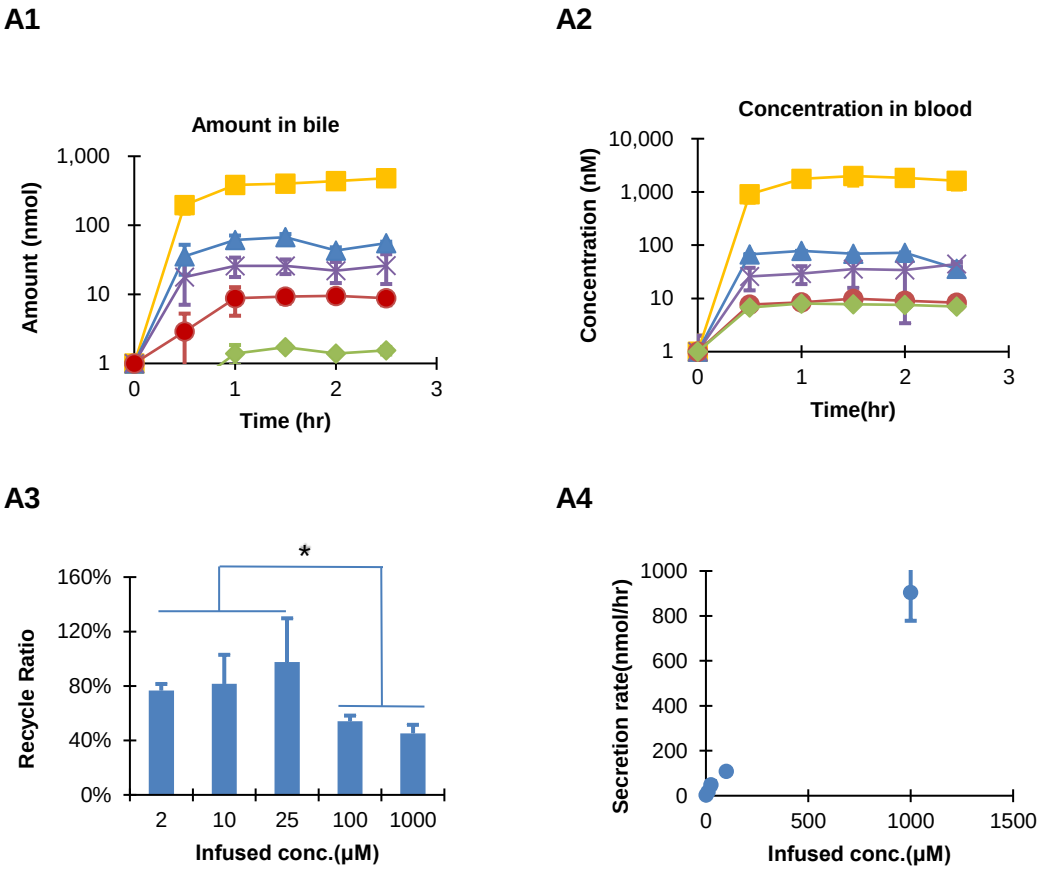
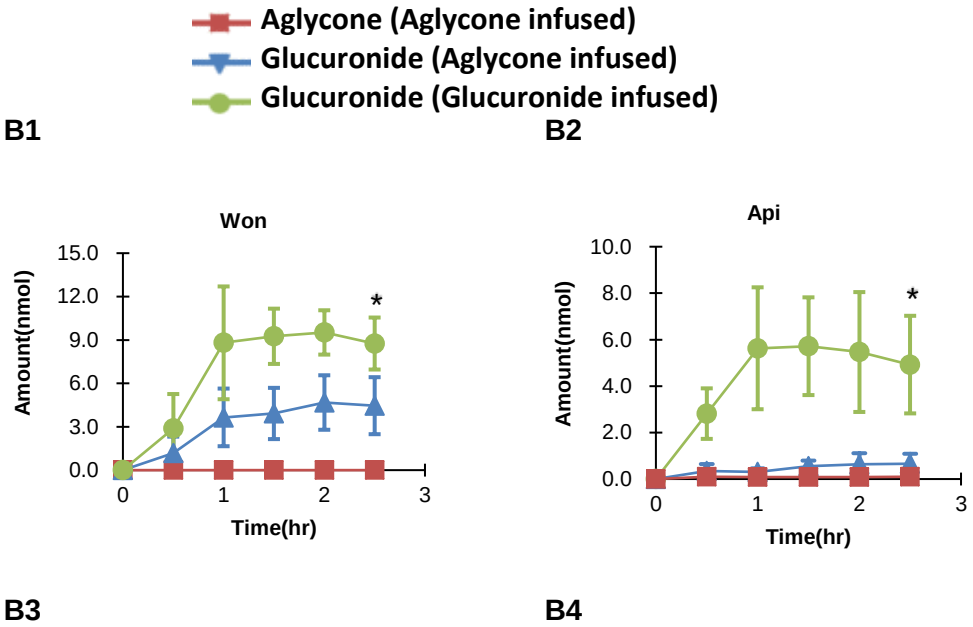


Fig.1A The concentration dependent infusion of wogonin-7-glucuronide (2μM to 1000μM) were summarized in Fig 1A. The bile amount (1A1), blood concentration (1A2) and recycle ratio (1A3) were summarized. Secretion rate was plotted against

infused concentration in 1A4. One-way ANOVA with Turkey post-hoc was used to calculate the statistical significance. * indicated statistical significance, $p<0.05$.

3.1.2 Portal vein infusion of aglycones and corresponding glucuronides.

Lower recycle ratio was found for 6 aglycone compounds (including 4 flavonoids, raloxifene and ezetimibe, Fig. 1B1-1B7) compared to their corresponding glucuronides. All 6 glucuronides showed a significantly higher RR% than their corresponding aglycones (Fig.1B1-1B7). Among the aglycones, wogonin had highest RR% (41%) when using wogonin-7-glucuronide as the species of recycling. Other aglycones showed even lower RR% (<10%). The lower RR% of aglycones in comparison with their glucuronides indicated that hepatic uptake of the glucuronides are more important than their aglycones in enabling recycling in the form of glucuronides.



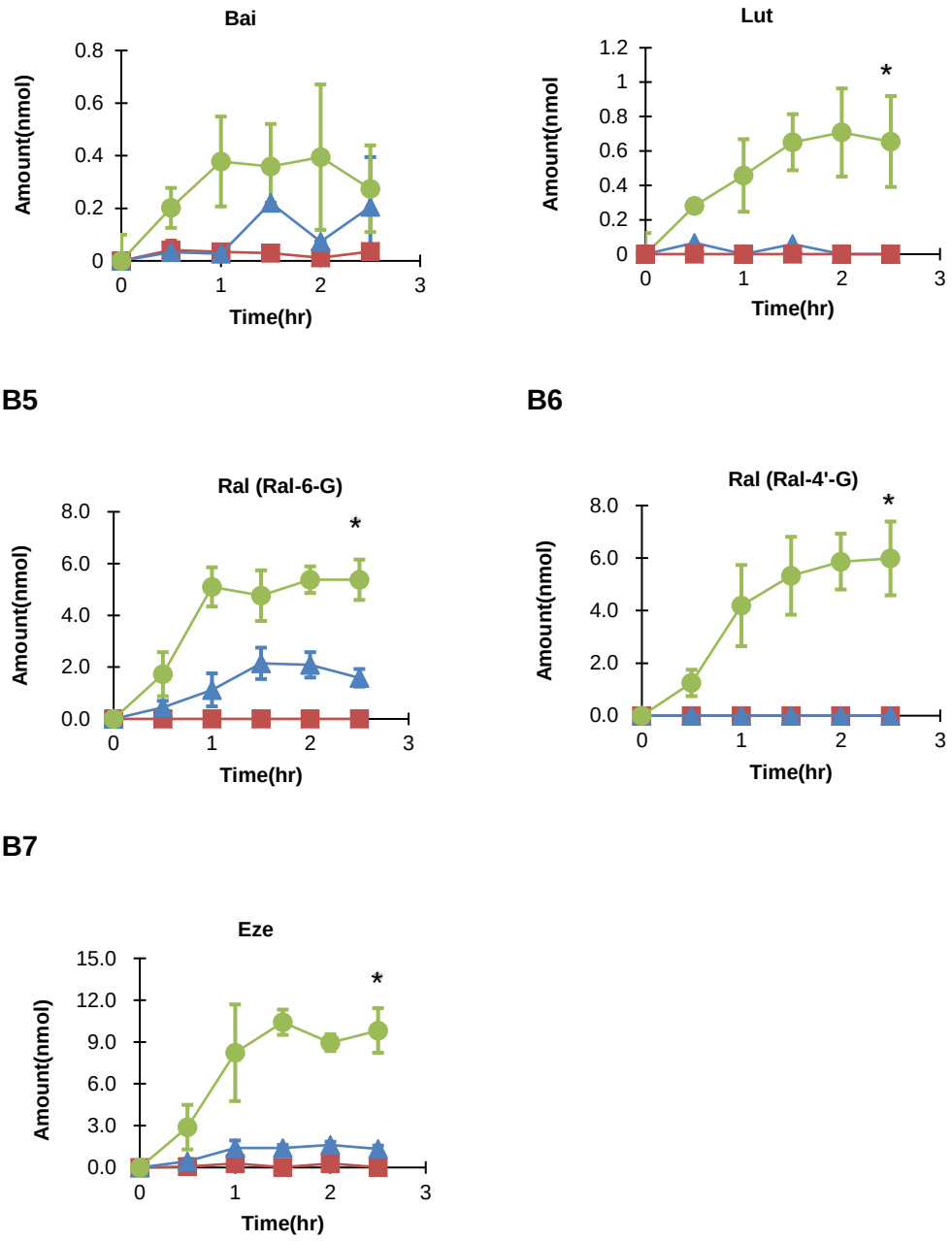
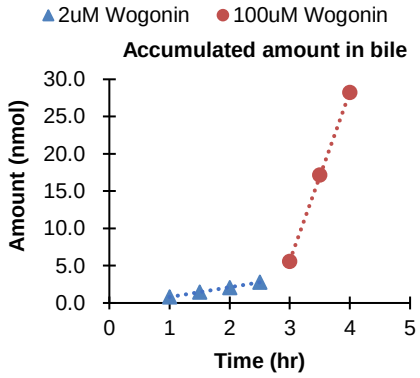


Fig. 1B Bile amount of glucuronides and aglycones after glucuronide/aglycone infusion were summarized in 1B1-1B7. Wogonin (1B1), apigenin(1B2), baicalein(1B3),luteolin (1B4),raloxifene (1B5,1B6), ezetimibe (1B7) and their

3.1.3 Concentration-dependent wogonin RR in a portal vein infusion study

To rule out the possibility that lower RR% value associated with aglycones was not due to extensive binding of wogonin to protein, we used higher concentration of wogonin, assuming higher concentration will result in more unbound wogonin. The results indicated that wogonin-7-glucuronide RR% is lower at the higher wogonin concentration (100 μM), (Fig. 1C1), with a decrease of 400% (~40% RR% at 2 μM vs ~10% RR% at 100 μM,Fig.1C3). The bile concentration of wogonin (aglycone) was below LLOQ at both concentrations. We don't think that this is due to enzyme saturation since the hepatic blood flow of rate is 15-20 ml/min, resulting in an instant dilution of infused compound (infusion rate is 2 ml/hr or 0.033 ml/min). The reported Km values for wogonin glucuronidation were ranged from 0.5 μM to 2 μM [98].

C1



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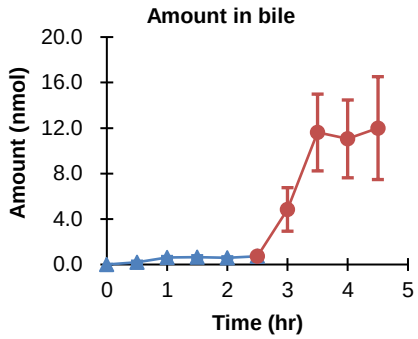


C3

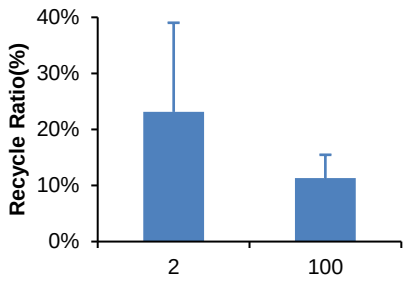


Fig.1C The wogonin infusion in 2 concentrations (2μM and 100μM) were summarized in Fig. 1C. Accumulated bile amount (1C1), bile amount (1C2) and recycle ratio (1C3) at 2 concentrations were summarized.

3.1.4 Portal vein infusion study with different flavonoid glucuronides

We were able to determine the RR% of 16 phenolic-O-glucuronides, derived from dietary phenolics and phenolic drugs (Table.S1). The results showed that RR% was highly variable, ranging from 95% to 5%. In a study of more than 7-O-glucuronides of flavonoids, we were able to show that 7-position of glucuronide alone is not sufficient to determine if a glucuronide will have high RR%, suggesting that the aglycone structures are important determinant of its RR%. This is rather interesting since there is only small structural differences between these flavonoid aglycones. In addition, glucuronides of drug molecules showed similarly large difference in their RR%, and their structures are quite different.

3.2 Cellular Uptake Studies Using OATP Overexpressing Cells

Portal vein infusion studies have showed that uptake is concentration-, structure- dependent and RR% of glucuronides was higher than corresponding aglycones, consistent with the notion that uptake is mediated by uptake transporters. Cell culture studies were performed to determine which transporters are important for their uptake. We chose to focus our initial effort on hepatic OATP transporters, because estradiol-O-glucuronide was taken up by hepatic OATP1B transporters [99, 100]. In addition, some of the results we obtained earlier had used isoflavone-glucuronides, where isoflavones are also called phytoestrogens. Cell studies are focused on OATP 1B1/1B3/2B1 cells because they are uptake transporters expressed on the basolateral side (blood side) membrane of hepatocytes.

3.2.1 Uptake of glucuronides in OATP 1B1/1B3/2B1 cells.

We found that glucuronide uptake in OATP cells varied greatly between various glucuronides (Fig.2A). And most glucuronides with high RR% was also taken up rapidly in these cells. However, E2G and luteolin-3'-glu were found to have low recycle ratio even though they were rapidly taken up by the OATP1B1-overexpressing HEK293 cells. An analysis of the bile samples found that three flavonoid glucuronides (luteolin-3'-glucuronide, icaritin-3-glucuronide and icaritin-7-glucuronide) were further metabolized into methylated glucuronide for luteolin-3'-glucuronide or became diglucuronodies for icaritin mono-glucuronide. In other words, for these 3 glucuronides, excretion was not the dominant elimination pathway.

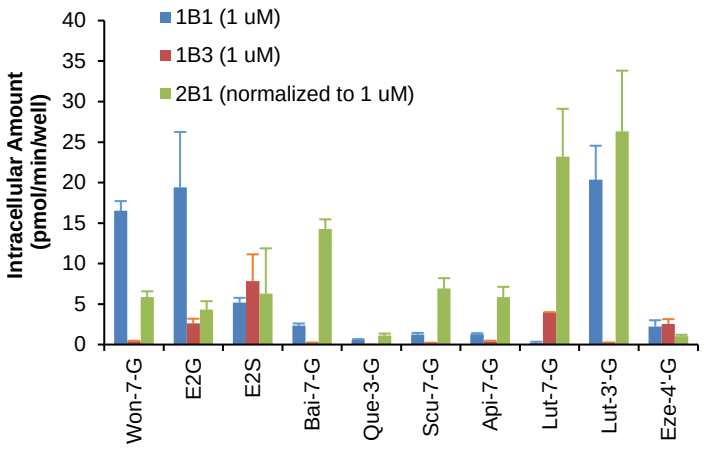


Fig. 2A Intracellular concentrations of different glucuronides in 3 over-expressed cell lines were summarized in Fig.2A.

3.2.2 Concentration-dependent uptake of glucuronides in OATP1B1/1B3/2B1 cells.

We determined the K_m and V_{max} values of 3 flavonoid glucuronides as representatives of high (wogonin-7-glucuronide), medium (apigenin-7-glucuronide) and low (luteolin-3'-glucuronide) RR% as showed in Fig.2B1 to 2B4 and Table 1. We found that these glucuronides have good affinity to OATP1B1, 1B3 or 2B1 with K_m values in the several micromolar range (Table 1), well within their *in vivo* exposure levels reported in the literatures [101, 102]

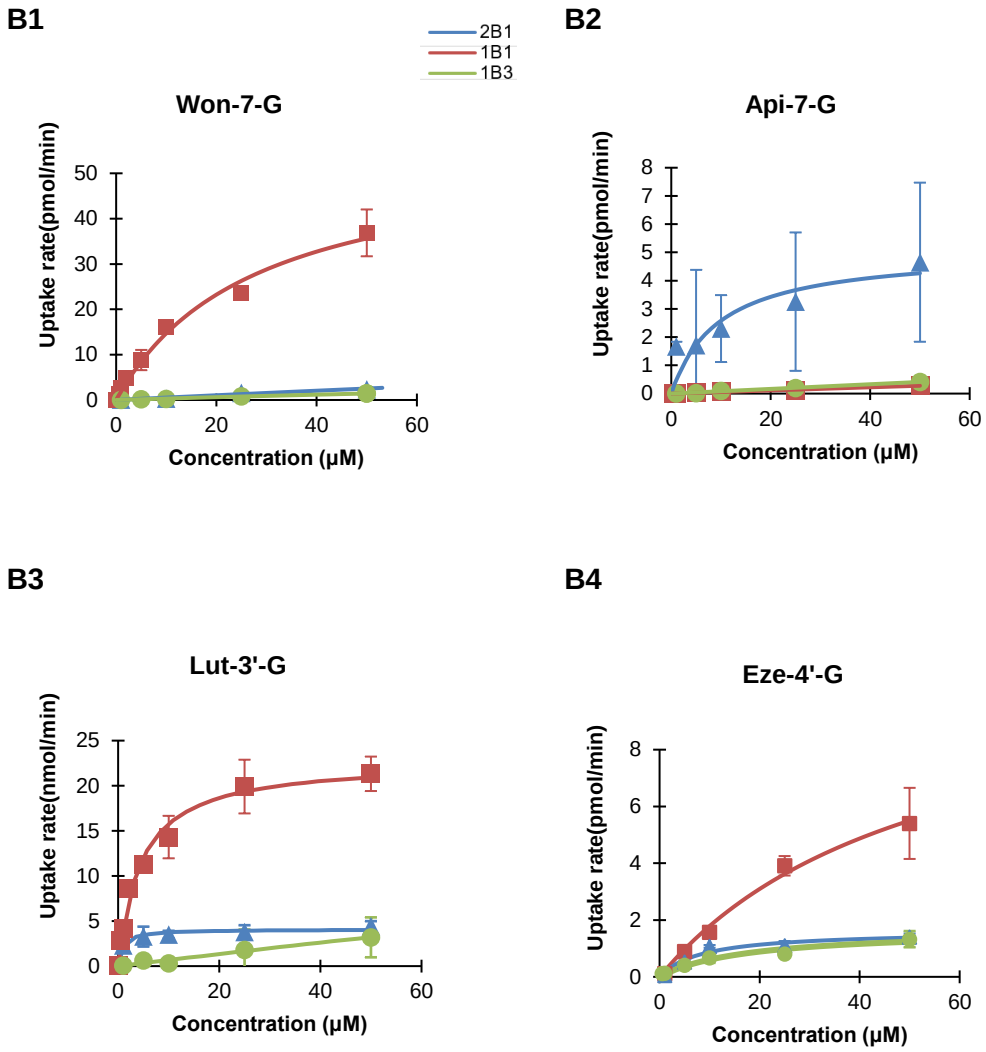


Fig.2B Cell uptake rates of wogonin-7-glucuronide (B1), apigenin-7-glucuronide (B2), luteolin-3'-glucuronide (B3) and ezetimibe-4'-glucuronide (B4) at different concentrations on OATP1B1/1B3/2B1 over-expressed cell lines.

Compound	1B1		1B3		2B1	
	Km(μM)	Vmax (pmol/min)	Km(μM)	Vmax (pmol/min)	Km(μM)	Vmax (pmol/min)
Won-7-G	27.68	53.37	>50	11.72	>50	55.26
Lut-3'-G	4.46	22.79	>50	9.62	0.89	4.07
Api-7-G	>50	>200	>50	>200	9.72	5.09
Eze-4'-G	>50	11.45	17.64	1.67	8.55	1.61

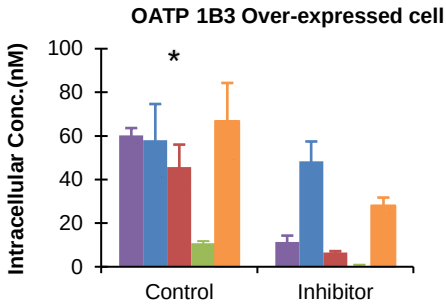
Table.1 Kinetic parameters of uptake (Km and Vmax) of selected glucuronides wogonoside, luteolin-3'-glucuronide (Lut-3'-glu) and apigenin-7-glucuronide (Api-7-glu) in OATP1B1/1B3/2B1 over-expressed cells

3.2.3 Inhibition of glucuronide uptake in OATP 1B1/1B3/2B1 cells.

Rifampicin (25 uM) was shown to be potent inhibitor (>90% inhibition) of uptake of OATP1B1 and OATP 1B3 substrate E2G, wogonin-7-glucuronide, luteolin-3'-glu (all at 1 uM) (Fig.2C1 to 2C3), consistent with literature-reported values for rifampicin when it was reported to have strong pharmacokinetic interaction with atorvastatin and provastatin (classical OATPs substrate) in healthy volunteers [103, 104]. In addition to rifampicin, telmisartan was also shown to be a potent inhibitor (>90%) of uptake OATP 2B1 substrate estrone-3-sulfate and luteolin-3'-glu.

C1

C2



C3

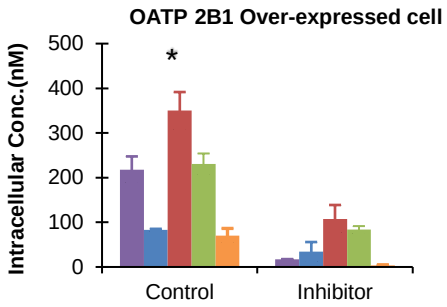


Fig. 2C The intracellular concentrations of 5 glucuronides with/without the present of specific transporter inhibitor in OATP1B1 (C1), OATP1B3(C2) and OATP2B1(C3) were summarized in Fig. 2C. Statistical significance was calculated by using student *t* test. * indicated $p<0.05$. Significant differences existing with/without present of inhibitor.

3.2.4 Cross-inhibition experiments in OATP 1B1 cells using glucuronides and their corresponding aglycones.

To determine if a non-substrate would be an inhibitor of glucuronide uptake in OATP1B1 cells, we employed several known substrates and non-substrates of OATP1B1, and showed that non-substrates were not inhibitors of substrate

uptake into the cells (Fig.2D1 to 2D3). At the same time, the results indicated that the inhibition capability of the aglycones were higher than their corresponding glucuronides, which is different from what was observed for difference in RR% (higher RR% for glucuronides than aglycones in general).

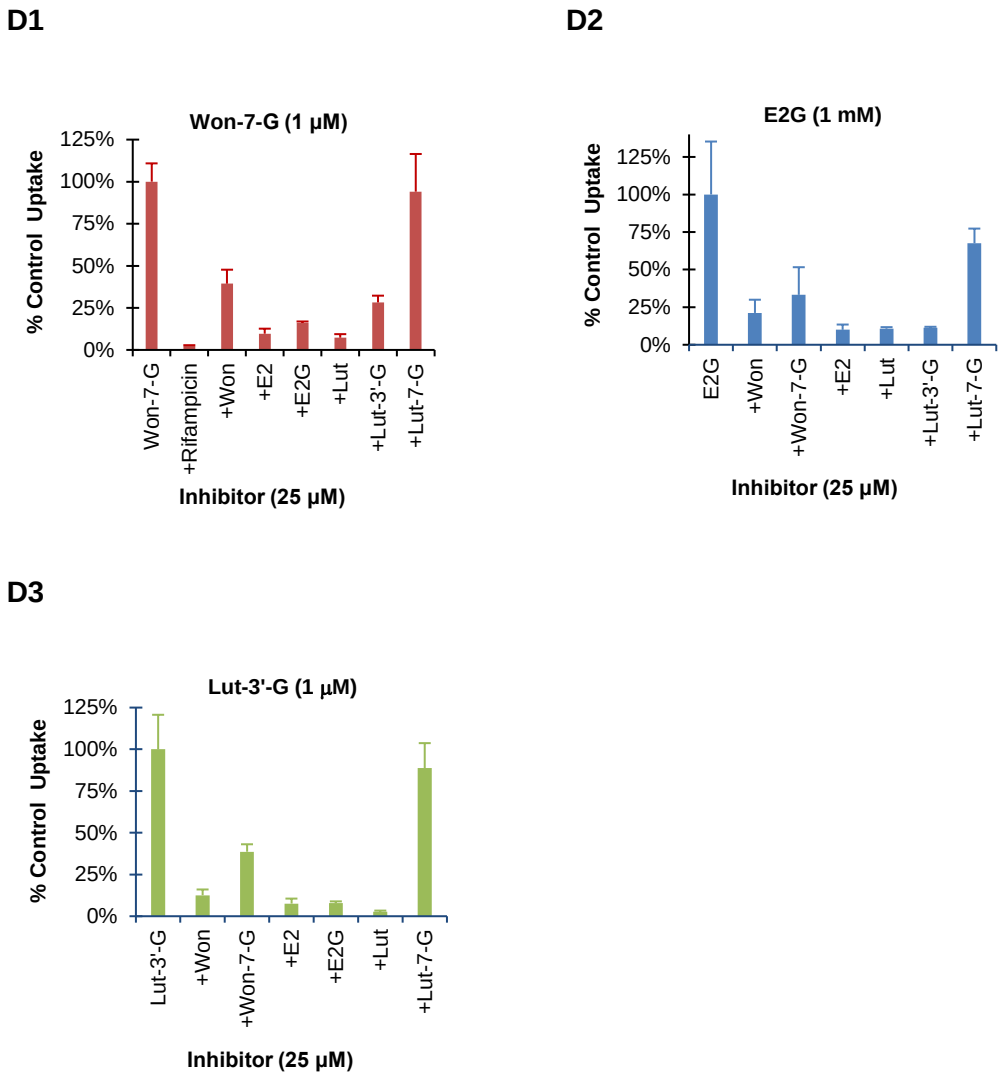


Fig 2A Percentage of inhibition of 3 glcuronides using different aglycones and glucuronides as inhibitors were summarized in Fig.2D. Wogon-7-glucuronide (2D1),

estradiol-17-glucuronide (2D2) and luteolin-3'-glucuronide (2D3) were selected as substrates. Other glucuronides and their corresponding aglycones were applies as inhibitors.

3.3 Pharmacokinetic study of wogonin/baicalein and ezetimibe/acetaminophen.

The blood concentration-time curve of wogonin-7-glucuronide, baicalein-7-glucuronide, acetaminophen-glucuronide and ezetimibe-4'glucuronide were summarized in supplement Fig. S1A to 1D. The elimination half-life were calculated by WinNonlin using two-compartment model. The wogonin/baicalein pair was selected as one of them (wogonin-7-glucuronide) has high RR% and the other one has very low RR%. The same can be said of ezetimibe-4'-glucuronides (high RR%) and acetaminophen glucuronide (low RR%).

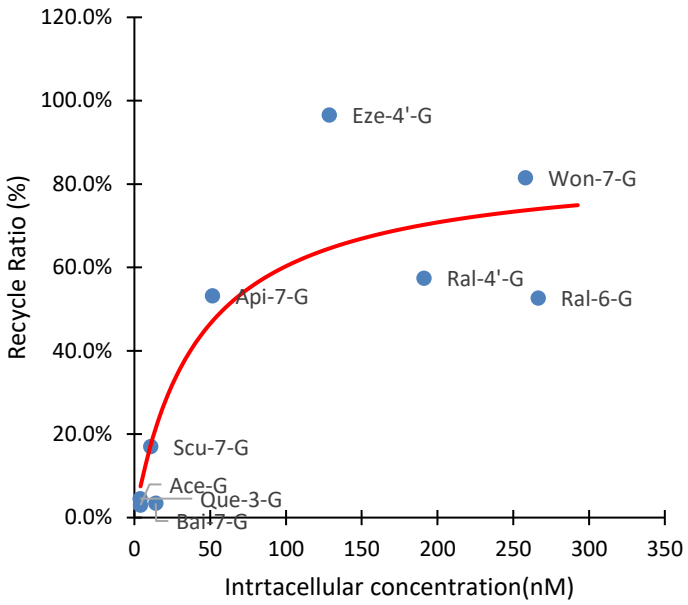
3.4 Correlation of recycle ratio, cell uptake and elimination half-life.

A total of 66 X,Y,Z combinations were tested by Fisher exact test (Supplement table S2). The results indicated that 41 out of 66 combinations showed significant correlations. Including physiological relevant combination (close to the protein expression level in human liver, where X=6, Y=2, Z=2), intracellular concentration showed significant correlation with recycle ratio. 25 out of 66 combinations did not show significant differences. For the 25 combinations, 18 of them did not have physiological relevance. In these combinations, the weighting of OATP1B1 was less than 2, while OATP1B1 was the most abundant

[105, 106] and most important uptake transporter expressed in liver (based on the In Vitro Metabolism and Transporter Mediated Drug-Drug Interaction Studies Guidance for Industry published by FDA). 7 out of the 25 combinations gave OATP1B3 a high weightings (Y>4), which is not consistent with physiological condition. The expression level of OATP1B3 was not as high as OATP 1B1 in human liver [107, 108]

Taken together, these results demonstrated there were solid correlation between cellular concentration and RR%.

This correlation appears to be a saturable process (Fig. 3) using an E_{max} model (Emax 86%±17%) with an EC_{50} value of 42 nM. In a limited dataset of four glucuronides with reported glucuronide half-life in rats, a glucuronide's elimination half-life was longer if the RR%was higher (supplement Figure S2). The results are consistent with the notion that hepatic uptake is the rate-limiting-step in the recycling of glucuronides.



Km	42.25±33.28
Vm	0.86±0.18
R^2	0.79
AIC	-10.08
SC	-9.68

Fig.3 The result of using recycle ratios and intracellular concentrations to fit with Emax model was summarized in Fig.3.

3.5 Comparison of RR in Male vs Female Rats.

The results indicated that the RR% had no significant changes in different sex of animals with respect to wogonin-7-glucuronide and luteolin-3'-glu. The RR% of wogonin-7-glucuronide and luteolin-3'-glu in female rats do not have significant differences when compared with male rats (Supplement table S4). Interestingly, the RR% of baicalein-7-glucuronide increased from ~4% to ~10%. .

3.6 Comparison of Bile Secretion in Intestine Perfusion versus Portal Vein Infusion.

The results indicated that the bile secretion rates of these glucuronides had no significant differences in small intestine perfusion and hepatic glucuronide infusion. The secretion rates of small intestine perfusion are significantly higher than those in hepatic aglycone infusion (Table 2). The hepatic portal vein blood concentrations of glucuronides are also higher than tail vein blood (Fig. 3). Therefore, small intestine serves as an important metabolism organ and these perfused aglycones have been extensively metabolized and underwent recycling.

Dosing method	Hepatic glucuronide infusion	Hepatic aglycone infusion	Small intestine aglyconeperfusion
Infused compounds	Eze-4'-G	Ezetimibe (Eze)	Ezetimibe (Eze)
	Won-7-G	Wongonin (Won)	Wongonin (Won)
	Ral-6-G	Raloxifene (Ral)	Raloxifene (Ral)
	Api-7-G	Apigenin (Api)	Apigenin (Api)
	Bai-7-G	Baicalein (Bai)	Baicalein (Bai)
Measured compound	Bile secretion rate(nmol/hr)		
Eze-4'-G	19.31±1.85	2.93±0.41**[a,b]	21.94±5.29
Won-7-G	16.30±4.28	8.46±3.93**[a,b]	17.90±11.96

Ral-6-G	10.53±1.51	3.91±0.82**[a]	4.00±0.68
Api-7-G	10.64±4.49	1.22±0.76**[a,b]	11.32±4.08
Bai-7-G	0.69±0.29	0.09±0.07**[a,b]	ND[b]
Measured compound	Recycle ratio(%)		
Eze-4'-G	96.54±9.23	2.93±0.41**[a,b]	91.42±22.04
Won-7-G	81.5±21.41	8.46±3.93**[a,b]	74.58±49.83
Ral-6-G	52.64±7.54	3.91±0.82**[a,b]	16.67±2.83
Api-7-G	53.21±22.44	1.22±0.76**[a,b]	47.17±17.00
Bai-7-G	3.43±1.46	0.09±0.07**[a,b]	ND[c]

a. Significant difference between hepatic glucuronide infusion and hepatic aglycone infusion, $p<0.01$

b. Significant difference between hepatic aglycone infusion and small intestinal perfusion , $p<0.01$

c. Not determined due to below quantification limit.

Table 2. Comparison of bililary secretion rates and recycle ratios of glucuronides following hepatic glucuronide infusion, hepatic aglycone infusion and small intestinal aglycone perfusion. The rate of hepatic infusion was 20 nmol/hr and the rate of intestinal perfusion was 24 nmol/hr

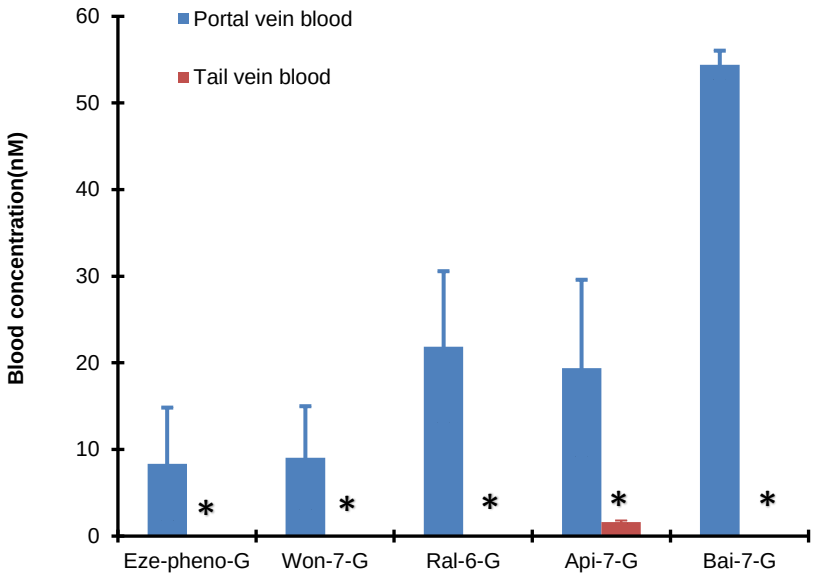


Fig.4 Portal vein blood and tail vein blood concentration of glucuronides. Statistical significance was calculated by using student *t* test. * indicated $p<0.05$.

3.7 Portal Vein Infusion Studies in the Presence of OATP Inhibitors

After infusion of OATP transporter inhibitors (rifampicin, telmisartan, E2G and E1S at 1000 μM each), the RR% value of wogonin-7-glucuronide decreased from over ~80% to ~40% and that of luteolin-3'-glu from ~7% to ~3% (Fig.5A to 5D). The results indicated that OATP transporters played the major role in the recycle of wogonin-7-glucuronide and luteolin-3'-glu, which is consistent with the results from cell uptake study. However, since we could not inhibit the

RR% further, other uptake transporters could also participate in the recycle of the glucuronides.

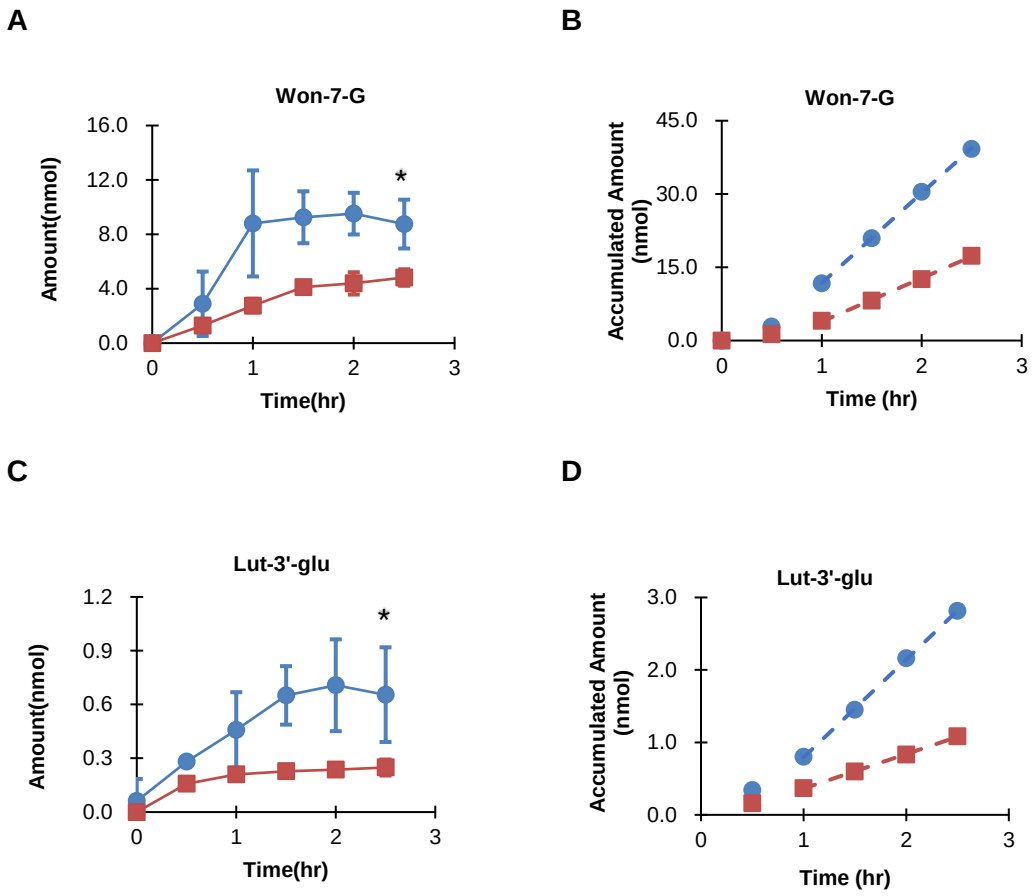


Fig. 5 Wogonin-7-G bile amount (A) and accumulated bile amount (B) were summarized as long as luteolin-3'-G bile amount (C) and accumulated bile amount (D) with/without the infusion with OATPs inhibitors. Bile amount was significantly

decrease with the present of inhibitors. Statistical significance was calculated by using student *t* test. * indicated $p<0.05$.

3.8 Impact of Cassette Dosing on RR of Glucuronides.

To rapidly determine the RR% of different glucuronides, we determined if a mixture of glucuronides could be given simultaneously to determine the excretion rate in a portal vein infusion study. We found that when given at a relative low concentration (10uM), glucuronides mixture didn't show a significantly different RR% values when compare to each glucuronide given individually (Supplement Fig. S3A to 3D). The results indicated that the recycle system is capable of handling multiple compounds in low concentration and the cassette dosing strategy could be applied to rapidly determine the RR% of different glucuronides present in a mixture.

DISCUSSION

We have defined a new disposition process as “Hepatoenteric Recirculation or Recycling (HER)” for phenolic drugs and phytochemicals (e.g., flavonoids). HER differs from EHR associated with bile acids and certain drugs, in that intestine is the main metabolite forming organ and liver is the recirculation organ. Historically, EHR of bile acids and various drugs [1, 3] is associated with metabolites that are generated in the liver and recirculation or recycling that occurs in the intestine. This new definition for the first-pass metabolism of phenolic drugs and phytochemicals pinpoints where the metabolites are formed and subsequently recirculated or recycled. In other words, the roles of liver and intestine are reversed in HER vs EHR. We define that the word “Recirculation” in HER refers to the fact that certain phenolic glucuronides uses liver for recirculation via bile. We also used the word “Recycling” in HER because glucuronides are not well absorbed in the intestine and these glucuronides have to be activated (i.e., made available again) by the microflora β-glucuronidases present in colon and terminal ileum to aglycones before re-absorption can occur.

The new definition is needed because: (1) unlike EHR of bile acids, intestine, not liver, is the major production organ of the circulating metabolites (i.e., phenolic glucuronides); (2) unlike EHR of bile acids, liver, not the intestine, is

the circulation organ; and (3) unlike EHR of bile acids, hepatic (e.g., OATPs) but not intestinal uptake transporters (e.g., ASBT) appears to be the rate-limiting step in determining the RR in HER. Due to the presence of HER, hepatic transporters (e.g., OATPs) will often determine the plasma concentrations of glucuronides, and play a much more important role than the relevant hepatic enzymes (e.g., UGTs) in determining how much glucuronides are in the bile and participate in recycling. For orally administered phenolics such as wogonin and ezetimibe with high RR, HER means that they will have double peaks in their plasma profiles (Fig.S4A to 4D) even though biliary metabolites are not generated by the liver (hence no EHR). For phenolics with more moderate RR, the role of metabolite generating organ or recycling organ is not mutually exclusive in HER vs EHR, in that biliary glucuronides may come from metabolites generated in the liver and intestine, with variable contributions depending on the compounds.

How significant is this new disposition process called HER in determining how phenolics and their glucuronides are dispositioned? We believe that HER is critically important in determining an orally administered phenolic's pharmacokinetic profile because a high RR means that a phenolic could have double peaks in their plasma profile and a longer terminal half-life, provided luminal metabolite excretion is not a major elimination pathway. The latter may translate into high plasma levels of aglycone if glucuronides are readily

hydrolyzed back into aglycones [109]. In other words, for extrahepatically generated glucuronides, HER is critically important because almost every glucuronides we tested were more likely to be in the bile if they are given as glucuronides vs corresponding aglycones, when infused directly via the portal vein (Fig. 1B1 to 1B7). If HER is critical, how important is EHR in the recycling process? The answer to this question is not that complex but the reasons are more convoluted. If an aglycone is readily taken up by the hepatocytes, as in the case of wogonin, raloxifene and ezetimibe, EHR will have significant contribution if enough aglycones leave intestine intact. In reality, these three compounds are nearly fully (>80%) metabolized after the aglycones are absorbed (Fig.4), and little is left for hepatocytes to fully contribute their metabolic capabilities. Therefore, we believe that HER is much more important than EHR for certain orally administered phenolics, whose major metabolic pathway is rapid glucuronidation, in determining their pharmacokinetic profiles and terminal elimination. It is possible that certain orally administered phenolics will have more contribution from EHR than HER, but our data did not have evidence in support of this.

We have proposed the new HER definition to delineate the significance and needs to describe the recycling of phenolics using a terminology different from EHR. How would HER impact the beneficial effects of dietary phenolics, therapeutic efficacy of phenolic drugs, and toxicities of phenolic toxins that are

taken orally, produced by microbiota or formed as the result of phase I metabolism in the liver? The possible answers are made of two parts, and subsequently combined to argue for the importance of adding HER for orally administered phenolics with extrahepatic glucuronidation as its major metabolic pathway.

Firstly, HER will shuttle more phenolic compounds and their metabolites, both beneficial and toxic, to the colon. This is because there is no known mechanism that can rapidly take up glucuronides in the small intestine. For compounds with beneficial effects including therapeutic effects, this can be used to prevent or treat diseases in the colon. The prevention or treatment effects can be achieved via a direct effect (e.g., suppress inflammation) or indirect effect (e.g., production of beneficial microbial metabolites that suppress inflammation). Alternatively, some of the toxic phenolic metabolites can be further biotransformed by the microbial enzymes to more potent toxins [110]. Taken together, the presence of HER for a phenolic compound could substantially enhance its colonic impact and maintenance of a healthy colon microbiota (i.e., a desirable colon microbiome homeostasis) that is essential for our health. Therefore, the use of phenolics to maintain and improve the health of microbiota is an important future research goal. On the other hand, HER could promote the recycling of toxic phenolic metabolites, enhancing its exposure and toxicities, and this should be limited.

Secondly, it may provide the theoretical foundation to understand a myriad phenomenon when there are drug-drug interactions involving glucuronidation. For example, if we only recognize the importance of EHR, if would be difficult to understand why inhibition of hepatic UGT does not always increase aglycone concentration and decrease glucuronide concentration in the blood. If we recognize that a phenolic is mainly processed by HER, an inhibitor that only inhibit liver UGT is not expected to affect plasma concentrations of orally administered aglycone and its glucuronides as long as intestine is the metabolite forming organ. In contrast, the emerging role of OATP means that the plasma concentrations of both aglycone and its glucuronide (if both are substrates of OATP) or just glucuronides (if only glucuronides are substrates of OATP), could increase by a drug that did not affect intestinal or liver UGT enzymes. Furthermore, one can proposed a variety of drug-drug interaction schemes, where only efflux transporters are affected, which could also raise moderately the plasma aglycone levels but could substantially increase the plasma glucuronide levels as inhibition of efflux transporters could force a redistribution of the glucuronides even though UGT activities in the intestine or liver did not change much[111]. Therefore, the proposed HER mechanism should help us determine the main mechanism by which drug-drug interactions occur to influence the plasma concentrations of aglycone and its glucuronides. Since the glucuronides are more variable in many instances, the impact of

glucuronidation-mediate drug-drug interaction will be greater when the glucuronides are pharmacologically active compounds like ezetimibe and dabigatran [112, 113] or can inhibit CYP enzymes such as gemfibrozil and clopidogrel. [114-116].

When combined, the new HER definition could help predict the potential drug-drug interactions under pathological conditions. Since small intestinal UGTs play a significant role in the HER of the compounds, factors that alter the expression of small intestine UGT expression will significantly influence the recycling of the compounds and their vivo exposure. Searching in Google Scholar by using key words combination “UGT+intestine+disease”, yielded a total of 14,500 hits. Among the top 50 results ranked by relevance, 15 of them reported different causes leading to change in intestinal UGTs including diseases (colitis, Gilbert’s disease etc.), genetic polymorphism and drug-drug interactions. The results indicated that pathological conditions will greatly influence the intestinal UGTs expression level and thus change the in vivo exposure of a phenolic whose disposition is controlled by a HER process. Similar conclusion could be made on hepatic OATPs, for OATPs mediate the hepatic uptake step of glucuronides generated from intestinal metabolism. Using “OATP+liver+disease” as key words combination, a total of 14,000 hits were found and 21 out of top 50 results ranked by relevance reported the

hepatic OATPs expression changes due to disease like cholestasis, liver hepatitis and carcinoma.

We have obtained several lines of evidence in support of proposed HER definition, especially for phenolics such as wogonin and ezetimibe. First, our data showed that when infused directly into the portal vein, the vast majority of their glucuronides reached bile, displaying a high recycle ratio (RR) (Table S1). For both compounds, portal vein concentrations of glucuronides were much higher than the corresponding aglycone in the rat intestinal perfusion studies (Fig. 4). Second, direct portal vein infusion of aglycone was substantially less effective in producing biliary excretion of glucuronides than portal vein infusion of corresponding glucuronides (Fig. 1B1 to 1B7). Third, hepatic OATPs took up these glucuronides in a saturable process that can be inhibited by OATP inhibitors (Fig 2B, Fig.2C1 to 2C3). Four, the structural differences between phenolic-glucuronides had a substantial impact on the RR. Taken together, these results showed clearly that intestine is the glucuronide forming organ, and liver is the glucuronide circulating (recycling) organ for these two compounds. Hence, the term HER properly describes the determining disposition processes of these two compounds, one naturally occurring polyphenol flavonoid and a prescription drug, which share minimal structural similarity. The latter suggest that HER could be broadly applicable to other phenolic phytochemicals and drugs.

Although many glucuronides that are good substrate of OATP1B1/1B3/2B1 appear to have higher HER RR (Fig.3), we also have two instances (i.e., luteolin and icaritin) where they did not correlate. We found that these two glucuronides were further metabolized in the liver, rendering their mono-glucuronidation metabolites unavailable for biliary excretion. Whereas we have obtained large amount of evidence in support of roles of OATP uptake transporters in enabling HER, we could not rule out the contribution from other uptake transporters, since we could only achieve a maximum of 50% inhibition of wogonin-7-glucuronide RR% in portal vein infusion experiment. It is likely that transporters belong to the OAT3 subfamily contributed to their liver uptake, since several flavonoid glucuronides were shown to be OAT3 substrate [117], which is expressed in rat liver [118].

Lastly, we have significant but not overwhelming structural diversity in our phenolic glucuronides, and as such, we could not predict which structure will have high RR. The empirical evidence support the hypothesis that good uptake of glucuronides by the OATPs will correlate with excellent RR, as long as the glucuronides taken up by the hepatocytes are not further metabolized. Because the direct portal vein infusion method is convenient to use with cassette dosing possible (Supplement Fig.S3A-3D), HER potential can be readily estimated using OATP overexpressing cells and confirmed using the portal vein perfusion

model. The latter should allow medicinal chemistry to design new compounds that are tailored to treat intestinal, especially colonic diseases. In addition to this difficulty, the role of efflux transporters may further complicate the disposition behaviors of phenolics and their glucuronides, and this is duly noted here but we will not get into the role of efflux transporters for brevity purpose.

In conclusion, a new disposition terminology called Hepatoenteric Recycling or HER has been proposed to describe and explain the disposition of dietary phenolics and phenolic drugs that are taken orally with intestine as their major metabolism (i.e., glucuronidation) organ and liver as their major recirculating organ. The new terminology correctly captures the recycling of relevant phenolics, similar to the use of “Enterohepatic Recycling” to capture the recycling of bile acids, where the liver is the metabolism organ and intestine as the recycling organ. This new term HER more accurately capture the disposition process of phenolics are administered orally and can be used to better understand why this class of compounds may have larger than expected effects in the colon for human health and diseases. It may also help to delineate drug-drug interaction mechanisms involving intestinal UGT enzymes and hepatic transporters of glucuronides, a major challenge that we are still facing today because of all the complexity involved.

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Chapter 6 Conclusion and Future Directions

As a conclusion of the study, a LC/MS method was established and validated for biological sample analysis. A new concept called “Hepatoenteric Recirculation/Recycling (HER) was proposed to describe the recycling process where intestine is the organ for metabolite formation and liver is the organ for metabolite recirculation. The mechanism of how glucuronides participated in HER was partially elucidated. OATPs were identified and considered playing a critical role in the recycling of glucuronides. The contribution of individual isoform was evaluated. The hepatic uptake significantly correlated with the recycle ratio, which indicated that hepatic uptake was the rate-limiting step in the recycling process of phenolic glucuronides. Single dose and multiple dose PK studies were conducted to further confirm the in vivo impact of recycling. A new

In our study, we were able to show that certain flavonoid glucuronides and some phenolic drug glucuronides when infused into the portal vein directly are able to get recycled. The RR of these glucuronides was usually better than their corresponding aglycones. Several glucuronides of phenolic compounds, including flavonoids and clinical used drugs (raloxifene and ezetimibe), were able to recycled at high efficiency, where recycle ratios (RR %) were 50% or even higher (>80%). The small intestine perfusion results also indicated that over 80% of drugs including ezetimibe and raloxifene were absorbed in small intestine and nearly all the compounds were found as the glucuronide form in portal blood. Considering their highly efficient

metabolism in small intestine, it is clear that extrahepatic metabolism could be a major source of glucuronide formation in vivo. Highly RR% of the intestinal generated metabolites suggest that liver could also serve as the recirculating organ that mediates the uptake and excretion of glucuronides. Thus, our data provide evidence to support the major contribution of extrahepatically formed glucuronides to the EHR. Moreover, hepatic uptake appears to be the rate-limiting step in the EHR of extrahepatically formed glucuronides since there were strong saturable correlation between OATP cell uptake and RR.

We have successfully identified that OATP 1B1/1B3/2B1 playing a significant role in the hepatic uptake process of these glucuronides. The uptake rates of different glucuronides in OATP 1B1/1B3/2B1 were determined by OATP over-expressed cells. Different glucuronides indicated different Km values to individual isoform, which showed the specificity of the recycling system. 16 glucuronides were tested on both in vitro and in vivo models and the in vitro cell uptake results indicated a good correlation with the recycle ratio, which confirmed our hypothesis that hepatic uptake is the rate-limiting step.

Prolonged elimination-half life was also observed in compounds with higher recycle ratios in single dose PK study. In multiple dose PK study, increased AUC and colon concentration of compound with higher recycle ratio was also observed. It supported our hypothesis that in vitro data could be applied to estimate in vivo recycling, which set the foundation for further prediction of in vivo exposure by using in vitro data. Our results also showed that different glucuronides have preferred affinity to certain

specific OATP isoform, and that the OAPT-mediated uptake of glucuronides could be lowered by 50% in the presence of OATP inhibitors.

In conclusion, there is an efficient pathway for the hepatic recycling of metabolites generated outside hepatocytes. In this pathway, hepatic uptake is the rate-limiting step instead of hepatic metabolism and OATPs played a significant role which was confirmed by both in vitro and in vivo results. The OATP over-expressed cell uptake results were well correlated with the RR%. We proposed to give a new concept called Hepatoenteric recycling (HER) to describe the recycling of these extrahepatically generated phenolic glucuronides because it vastly different from traditional recognized enterohepatic recycling (EHR). In EHR, the hepatic metabolism is more important as glucuronides are formed in hepatocytes, while hepatic uptake is more important in HER since glucuronides are generated before reaching hepatocytes. Uptake transporters like OATPs play a critical role instead of hepatic metabolism enzymes in HER. The role of enterocytes and hepatocytes are switched in HER compared to EHR. In HER, intestine serves as the metabolism organ and liver serves as the circulating organ while liver serves as the metabolism organ and intestine serves as the recirculating organ in EHR. However, the function of metabolite generation or metabolite recycling is not necessarily exclusive. The HER process may have some hepatic metabolism of aglycones that escape from intestinal metabolism, whereas the EHR may have some metabolites generated in enterocytes and recycled by the hepatocytes. The latter may occur because bile excreted phase II conjugates could be hydrolyzed to generate aglycone in the lumen, and when the aglycone is re-absorbed, it could be metabolized in the enterocytes and metabolites will then reach liver.

This new term is proposed to describe the disposition pathway where orally dosed phenolic compounds and dietary phenolics are majorly metabolized in small intestine and recycled in liver. It helps recognize the contribution of phenolic metabolites that have the great potential of influencing the in vivo efficacy by participating in the recycling process as their aglycones. For example, clinically used drug raloxifene was reported to have 60% of intestinal absorption and relative extensive intestinal metabolism. Based on our results, over 50% of the raloxifene glucuronides are excreted into bile which indicated that the over 50% of raloxifene remained in the body as the form of metabolite. With hydrolysis and re-absorption process, raloxifene would have multiple in vivo exposure. Another example is cholesterol-lowering drug ezetimibe. It has active metabolites, which have similar pharmacological potential as the aglycone form. The recycling of the metabolites would greatly increase the in vivo efficacy of ezetimibe. The recycling of the active metabolites not only provided multiple in vivo exposure of aglycone, but also increase the hepatic exposure of active metabolites. As a result, the drug efficacy was greatly increased due to the increased exposure of both ezetimibe and its active metabolites.

The study also differentiates the metabolism organ in HER and EHR, which helps dose adjustment in clinical situation for patients with GI tract or liver diseases. For patients with impaired intestinal function like Crohn’s disease, ulcerative colitis and intestinal carcinomas, the intestinal metabolism is influenced while hepatic metabolism remains the same. Drug in vivo exposure could be greatly increased in this case. Ezetimibe was tested in our study and it was confirmed to have extensive intestinal metabolism and HER. The impaired intestinal metabolism indicated a high

potential for acute liver damage due to the increased exposure of ezetimibe in liver [119]. Therefore, the dose adjustment is necessary when considering the impaired metabolism capacity of patient with intestine diseases.

The new terminology helps complement the general understanding of enterohepatic circulation. Moreover, it is a more accurately description where the switched role of liver and small intestine in these two different recirculation schemes is captured. It provide an explanation why some phenolic compounds have larger in vivo impact than their in vitro activity. In addition, it helps delineate the mechanism of drug-drug interaction not only related to metabolism enzymes like UGTs, but also related to uptake transporters like OATPs. With diseases influencing UGTs or OATPs expression level, there is a potential of increasing in drug *in vivo* exposure. It makes our study of better significance in clinical situation.

However, the mechanism of HER is not fully elucidated. Since the inhibition of OATPs only inhibit 50% of the recycle ratio, there might be other uptake transporters like OAT, OCT participating in the process. In addition, the contribution of efflux transporters were not clearly considered in the HER process. The number of studied drugs were still limited. Only 16 phenolic glucuronides were studied so far including 13 flavonoid glucuronides and 3 drug glucuronides.

In our future study, we expected to identify other uptake transporters that may participate in the recycle process. In addition, the contribution of efflux transporters will be estimated. More phenolic drugs will be included to further enrich our current database. More pharmacokinetic study and pharmacodynamics study will be

conducted to correlate in vitro properties, recycle ratio and in vivo performance as a comprehensive model to predict the in vivo drug efficacy. Considering that the differences of chemical structure among all the flavonoids are pretty small but the recycle ratio ranges from 5% to over 80%, there must be a structure activity relationship existing that has a significant impact on their recycle ratios. Therefore, we also expected to establish the structure-activity relationship (SAR) that predict the recycle process based on the chemical structure.

As a long-time goal, we expected to establish a comprehensive model, which helps predict drugs' in vivo efficacy based on their in vitro properties. This model would incorporate both uptake and efflux transporters and metabolism enzymes. By differentiating the contribution in enterohepatic recycling and hepatoenteric recycling, this model will give a more accurate estimation about a phenolic drug's in vivo disposition. A more accurate and comprehensive understanding of enterohepatic recycling would also make it easier to better understand and evaluate the drug related interaction and toxicity.