FEXOFENADINE AND ORGANIC ANION TRANSPORTING POLYPEPTIDES (OATPs): TRANSPORT AND DRUG-DRUG INTERACTIONS

By

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ABSTRACT

Transporters play a major role in the absorption and disposition of fexofenadine, suggesting this drug could be used as a probe of transporter activity. When fexofenadine was administered in combination with four drugs (buspirone, caffeine, dextromethorphan and losartan) used to probe cytochrome P450 (CYP) activities, a significant decrease in fexofenadine AUC was observed without a change in elimination. Based on this observation, I hypothesized a fexofenadine-probe drug interaction was occurring during oral absorption, and that this interaction was occurring at an enterocyte-expressed OATP. This interaction was reproduced and studied using *in vitro* model systems.

In Specific Aim 1, a specific LC-MS/MS method was developed and validated for the quantification of fexofenadine and the other four probe drugs for use in the remaining specific aims. In Specific Aim 2, the interaction between fexofenadine and four enterocyte- and hepatocyte-expressed OATPs was characterized, and OATP1A2 was identified as the most effective transporter of fexofenadine, with a K_m of 35 μM. Because fexofenadine was efficiently transported by OATP1A2, the four CYP probe drugs were tested as inhibitors of OATP1A2-mediated fexofenadine transport in Specific Aim 3. Buspirone, losartan, and dextromethorphan each inhibited OATP1A2-mediated fexofenadine transport in a concentration dependent manner. This inhibition could explain the

decrease in fexofenadine oral bioavailability seen in the clinical study we had previously conducted. The replication of the fexofenadine-probe drug interaction in this model system supports the conclusion that OATP1A2 is the major uptake transporter for fexofenadine absorption in the enterocyte, and suggests that fexofenadine may be an effective probe drug for this transporter. In Specific Aim 4, I further characterized the fexofenadine-probe drug interactions using the three known OATP1A2 polymorphisms: Ile13Thr, Arg168Cys, and Glu172Asp. While the mutants functioned as expected with regard to fexofenadine transport, the presence of the mutation did not alter the observed drug-drug interactions seen previously with OATP1A2 and the CYP probes. Taking these data into account, it appears the fexofenadine-drug interaction seen previously is not affected by single nucleotide polymorphisms.

This work demonstrates that OATP1A2 is capable of transporting fexofenadine and that several CYP probe drugs inhibit its transport by OATP1A2. This latter observation limits the utility of fexofenadine to be used as a single probe, rather than as part of a probe drug cocktail.

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Table of Contents in Brief

Table of Contents Expanded

Chapter 1

Background and Significance

Chapter 2

Experimental Materials and Methods

Chapter 3

Quantification of the Transporter Substrate Fexofenadine in Cell Lysates by LC-MS/MS

Chapter 4

Fexofenadine Drug-Drug Interactions at Organic Anion Transporting Polypeptide 1A2

Chapter 5

Multiple Organic Anion Transporting Polypeptides (OATPs) Play A Role in the Disposition of Losartan

Chapter 6

OATP1A2 Single Nucleotide Polymorphisms and Their Effect on Fexofenadine Transport and Drug-Drug Interactions

Chapter 7

Summary and Discussion

List of Tables

List of Figures

List of Abbreviations

AUC	area under the curve
CHO	Chinese hamster ovary
C_{max}	peak concentration
CV	coefficient of variation
CYP	cytochrome P450
E3S	estrone-3-sulfate
E17-ßG	estradiol-17ß-glucuronide
EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionization
FBS	fetal bovine serum
HEK293	human embryonic kidney 293
HPLC	high performance liquid chromatography
IC_{50}	half maximal inhibitory concentration
IS	internal standard
K_m	Michaelis-Menten constant

LC-MS/MS	liquid chromatorgraphy-tandem mass spectrometry
LLOQ	lower limit of quantitation
MDR1	multi-drug resistance protein 1 (P-glycoprotein)
MRM	multiple reaction monitoring mode
mRNA	messenger RNA
m/z	mass/charge
OATP/Oatp	organic anion transporting polypeptide
PBS	phosphate buffered saline
P-gp	P-glycoprotein (MDR1)
QC	quality control
r^2	correlation coefficient
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SNP	single nucleotide polymorphism
SLCO	solute carrier family of the OATPs
$t_{1/2}$	half-life

t_{max}	time to peak concentration
V_{max}	maximal rate of transport
WT	wild-type

List of Appendices

Chapter 1

Background and Significance

Variability in drug response is a major clinical challenge that needs to be managed. Pharmacokinetics often contributes significantly to variability in drug response. There are two types or sources of variability in drug pharmacokinetics: *between-subject* or inter-individual variability and *within-subject* or intra-individual variability. Sources of inter-individual variability in drug pharmacokinetics may include genetic differences in drug metabolism, environmental factors, gender, age, and the presence of disease, whereas intra-individual variability in drug response can often occur as a result of hormonal changes, physiologic changes due to disease progression, or, more commonly, as a result of interactions with drugs or other chemicals. Experimental approaches to detect and investigate mechanisms of such variability in pharmacokinetics are essential tools for understanding, predicting, and managing such pharmacokinetic variability, and variability in drug response.

1.1: Probe Drugs and Probe Drug Cocktails

The use of probe drugs for measuring enzyme and transporter activity *in vitro* and *in vivo* is a valuable tool for the study of variability in pharmacokinetics (Rendic et al., 1997; Bjornsson et al., 2003; Petsalo et al., 2008). Safe, well-characterized, and validated probe drugs for many of the key

cytochrome P450s (CYPs) are available and in use (Frye et al., 1997; Streetman et al., 2000; Zhu et al., 2001, Christensen et al., 2003; Ryu et al., 2007). However, specific probe drugs for Phase 2 drug metabolizing enzymes or for transporters are not well-characterized nor are they routinely used. Probe drugs may be used individually; however the combination of multiple probe drugs into cocktails offers practical advantages, and now, becoming a more common approach. Compared to the administration of single probe drugs in multiple studies, the co-administration of multiple probes as a cocktail for drug metabolizing enzymes offers a more efficient mode of studying drug disposition and drug-drug interactions in humans (Breimer et al., 1990; Frye et al., 1997; Streetman et al., 2000; Zhu et al., 2001, Christensen et al., 2003; Ryu et al., 2007). Obvious advantages are minimizing the influence of inter-individual and intra-individual variability to drugs over time and the reduced cost of human subject studies. A probe drug cocktail containing buspirone, caffeine, dextromethorphan, and losartan was developed and used in a clinical study to examine the metabolic activities of CYPs in human subjects. As a novel approach, fexofenadine, which is not metabolized, was included as a fifth drug to assess its utility in characterizing membrane transporter functions in these human subjects.

Membrane transporters are proteins that transport drugs across cellular membranes and play an integral role in the absorption, distribution, metabolism, and excretion of drugs. Due to their important role in absorption and disposition,

changes in transport activity can ultimately result in alteration of the pharmacokinetics of drugs (Giacomini, et al, 2010; Müller et al, 2011; Zhang et al., 2011). A useful tool to study the role of transporters *in vivo* is the use of probe drugs. Fexofenadine, a safe, well-tolerated and approved drug, has been used as a probe of transporter activity both *in vitro* as well as *in vivo*. However, there is still uncertainty with regards to which membrane transporters influence fexofenadine pharmacokinetics and to what extent (Wang et al., 2002; Kharasch et al., 2005; Yasui-Furukori et al., 2005; Shimizu et al., 2006; van Heeswijk et al., 2006; Bailey et al., 2007; Glaeser et al., 2007; Robertson et al., 2008). Thus, it is critical to fully characterize the roles of various human transporters involved in fexofenadine pharmacokinetics and delineate their exact contributions, thus defining the utility of fexofenadine as a probe drug.

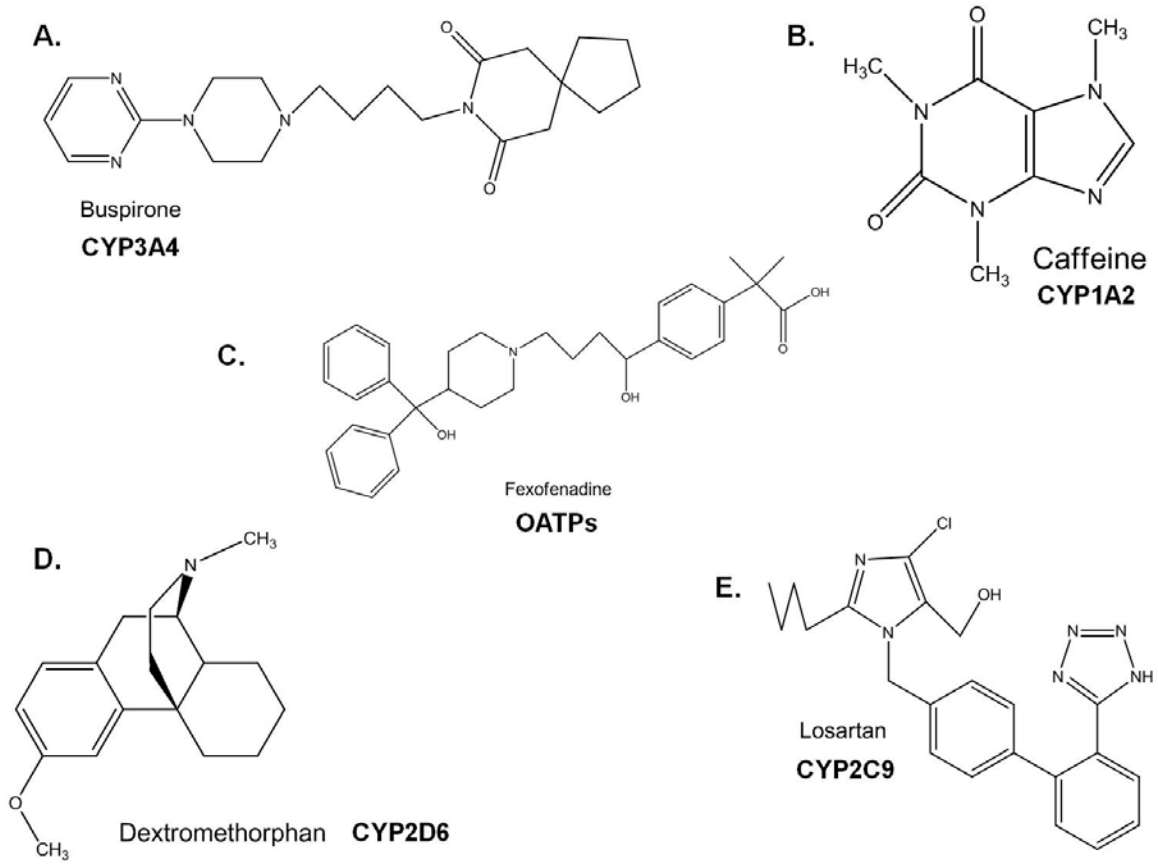
1.2: Components of The Kansas Cocktail

Probe drugs are pharmaceutical compounds that are often used as biomarkers of enzyme or transport activity. Probe drugs have been well defined for analyzing specific cytochrome P450 drug metabolizing enzymes but are not well established for assessing the role of membrane transporters in human subject studies. The Kansas Cocktail was designed and validated to assess the function of four key drug-metabolizing CYPs and to assess transporter function (Reed et al., 2010). The individual probe drugs and their targets are described and their structures are depicted in Figure 1.1.

1.2.1: Buspirone

Buspirone hydrochloride is an anti-anxiety agent that is not chemically or pharmacologically related to the benzodiazepines, barbiturates, or other sedative/anxiolytic drugs; it acts as a partial agonist at the 5-HT_{1A} receptor (Yocca, 1990). Although buspirone is almost entirely absorbed after oral administration, extensive first pass metabolism reduces its oral bioavailability to about 5% (Gammans et al., 1986). The principal route of metabolism of buspirone leads to the formation of its major metabolite, 1-pyrimidylpiperazine, which is present in plasma at a concentration higher than the parent compound (Jajoo, 1989). *In vivo* drug interaction studies have shown that the relative bioavailability of buspirone is increased by 3- to 19-fold by several well established CYP3A4 inhibitors, which suggests that buspirone is primarily metabolized by CYP3A4 (Kivistö et al., 1997; Lamberg et al., 1998; Laine et al., 2003). Accordingly, buspirone has been accepted an appropriate probe of CYP3A4 activity by the U.S. Food and Drug Administration (FDA 1999).

Figure 1.1:



The Kansas Cocktail. The five components of the Kansas cocktail are buspirone (A), caffeine (B), fexofenadine (C), dextromethorphan (D), and losartan (E). Buspirone is a probe for CYP3A4, caffeine is a probe for CYP1A2, fexofenadine is a probe for transporter activity, dextromethorphan is a probe for CYP2D6, and losartan is a probe for CYP2C9.

1.2.2: Caffeine

Caffeine is often used as a probe for CYP1A2 activity and was included in the probe drug cocktail for this purpose. The major metabolites of caffeine are 1-methylurate, 1-methylxanthine, 1, 7-dimethylurate, and 5-acetylamino-6-formylamino-3-methyluracil. Caffeine urinary metabolite ratios provide indices for activity of CYP1A2, and also of N-acetyltransferase (Frye et al., 1997; Scott et al., 1999; Streetman et al., 2000; Christensen et al., 2003; Ryu et al., 2007). Since direct measurement of caffeine clearance is cumbersome, the measurement of metabolite ratios of caffeine has been used instead as a noninvasive and easy way of indirectly assessing CYP1A2 activity (Peng et al., 2004).

1.2.3: Dextromethorphan

Dextromethorphan is a synthetic, non-narcotic, centrally-acting cough suppressant. The anti-tussive effectiveness of dextromethorphan has been demonstrated in both animal and human clinical studies and the incidence of toxic effects has been remarkably low. Dextromethorphan is rapidly absorbed from the gastrointestinal tract after oral administration (Moghadamnia et al., 2003). Dextromethorphan undergoes rapid and extensive first-pass metabolism mainly through the action of CYP2D6; the bioavailability of oral dextromethorphan ranges from 1 to 2% in subjects who are extensive

metabolizers and approximately 80% in those who are poor metabolizers (Capon et al., 2006). Its major metabolite is dextrorphan; allowing the ratio of dextrorphan/dextromethorphan to be used as a measure of CYP2D6 activity (Yu and Haining, 2001). CYP2D6 is a highly polymorphic gene with more than 100 known allelic variants (Abduljalil et al., 2010). Because the overall disposition of dextromethorphan is highly dependent on CYP2D6 activity, these polymorphisms are the main source of the wide inter-individual variation in the plasma levels attained and the response elicited by dextromethorphan (Gaedigk et al., 2008).

1.2.4: Losartan

Losartan is an angiotensin II receptor antagonist (Type AT_1) used to treat hypertension (Timmermans et al., 1993). It is primarily converted to its active metabolite E3174 by CYP2C9 in the liver (Stearns et al., 1995; Yun et al., 1995) and the metabolite is primarily excreted into bile (Dickson et al., 2003). Other sartan drugs such as valsartan and telmisartan are substrates of liver-specific OATP1B1 and 1B3 and losartan shares structural similarity (Yamashiro et al., 2006 and Ishiguro et al., 2006). Losartan, as a useful probe for CYP2C9 activity, is confounded by the fact that the drug is both passively and actively absorbed; therefore transporters may play a significant role in losartan disposition. Losartan has been used in various probe drug cocktails (Christensen et al., 2003; Ryu et al., 2007) and the ratio of E3174/losartan reflects CYP2C9 activity, however, the role of OATPs in losartan disposition is unknown.

There is a large inter-individual variation for CYP2C9 activity and resulting in altered clinical response to therapeutics metabolized by this enzyme (Miners and Birkett, 1998). This variation is most important for drugs possessing narrow therapeutic indices. There have been 556 SNPs found in the introns and exons of the CYP2C9 sequence (Wang et al., 2009). However, studies thus far did not reveal any association between CYP2C9 genotype and losartan AUC (Yasar et al., 2002; Lee et al., 2003). It appears that CYP2C9 polymorphisms affect losartan pharmacokinetics from a minor to moderate extent; because at higher exposure, CYP3A4 plays a larger role in losartan metabolism (>25 μM) (Lee et al., 2003). Pharmacokinetic interactions between fluvastatin and diclofenac (another CYP2C9 substrate) have been observed (Transon et al., 1995); but fluvastatin does not alter the pharmacokinetics of losartan (Meadowcroft et al., 1999). Fluconazole decreased the metabolism of losartan to E3174 (Kazierad et al., 1997; Kaukonen et al., 1998) by inhibition of both CYP3A4 and CYP2C9. However, itraconazole, a CYP3A4-specific inhibitor, had no significant effect on losartan oxidation *in vivo* (Kazierad et al., 1997; Kaukonen et al., 1998).

1.2.5: Fexofenadine

Fexofenadine is a widely used, non-sedating second generation H_1-receptor antagonist. While fexofenadine has been found to be a substrate for transporters, the antihistamine undergoes minimal metabolism in humans

(Lippert et al., 1995), thus making it a potential candidate for use as a probe drug for assessing transporter activity in human subjects.

1.3: Fexofenadine Pharmacokinetics

1.3.1: Fexofenadine *in vivo*

Lippert et al. (1995) conducted a mass balance and metabolism study of fexofenadine where six healthy male subjects were given a single oral dose (60 mg) of ^{14}C-fexofenadine. Recovery of radiolabeled fexofenadine was high, with 80% of the dose found in the feces and approximately 12% found in the urine. The majority of the dose (> 85%) was recovered as unchanged fexofenadine, indicating that metabolism is an insignificant elimination route for fexofenadine and that fexofenadine is either poorly absorbed and/or is secreted back into the gastrointestinal tract.

Stoltz et al. (1997) described the influence of food on the oral bioavailability of fexofenadine. Administration of fexofenadine contained in a solid oral dosage form (as a capsule or tablet), in the presence of a high fat breakfast, resulted in decreases in fexofenadine AUC and C_{max}, but not $t_{1/2}$ or t_{max}. Although the high fat breakfast was associated with decreases in extent of oral absorption, the magnitude of effect was much smaller than those for other

second generation antihistamines. As a result, administration of fexofenadine on an empty stomach is not required.

Russell et al. (1998) conducted a fexofenadine single- and multiple-dose pharmacokinetic and pharmacodynamic study in healthy male volunteers. The purpose of this fexofenadine study was to establish a maximum tolerated dose, to characterize the single- and multiple-dose pharmacokinetics, and to characterize the pharmacodynamics of its antihistamine activity. Fexofenadine was well tolerated at single-doses from 10 to 800 mg and at multiple-doses of 20 to 690 mg for 28.5 days. Mean C_{max} for a single dose of fexofenadine dosed as an oral solution ranged from 46-6383 ng/mL and occurred one hour after dosing. The apparent elimination ranged from 7.7 to 14 hours after dosing, with oral clearance being approximately 30-55 L/hr for a single dose of fexofenadine. For multiple dose studies of fexofenadine, steady state concentrations were reached 5 days after twice daily dosing, with C_{max} ranging from 58-4677 ng/mL. Similar to that observed following single doses, the fexofenadine apparent elimination half-life ranged from 8.8 to 12.9 hours. Steady state mean oral clearance was 26-49 L/hr and appeared to be independent of dose.

1.3.2: Fexofenadine *in vitro*

Transporters are involved in the pharmacokinetics of fexofenadine, thus, it is important characterize the roles of various human transporters involved in the

absorptive and distribution processes of this probe drug before being able to define its utility as a probe drug. Cvetkovic et al. (1999) determined the uptake kinetics of fexofenadine by human OATP1A2 in transiently transfected HeLa cells. The *in vitro* K_m was determined to be 6 μM, with a V_{max} of 58 pmol/mg protein/min (Cvetkovic et al., 1999). They also demonstrated that P-glycoprotein was able to efflux fexofenadine using basolateral to apical transport in an LLC-PK1 cell line overexpressing P-glycoprotein. Fexofenadine is also transported *in vitro* by OATP1B3 with a K_m of 108 μM (Shimizu et al., 2005), and one report suggested transport by OATP2B1 (Nozawa et al., 2004), however the signal appeared not to be statistically significant.

1.4: Membrane Transporters Involved in Drug Absorption and Disposition

After an orally administered drug undergoes dissolution within the gastrointestinal tract, it crosses the intestinal wall, reaches the liver via portal blood flow. Drug which is absorbed and escapes metabolism by the liver during this first pass through, will subsequently enter systemic circulation and distribute to the various tissues and organs throughout the body. Drug elimination, is comprised of drug metabolism, most often primarily via the liver, and by excretion of parent drug and metabolites into bile or into urine. Absorption, distribution, metabolism and excretion require that drugs pass through several biological membranes. The extent of drug transport across these membranes is affected by the physicochemical properties of a drug, such as size, lipophilicity, charge, or

degree of ionization. In addition, membrane transporters have a significant role in facilitating or preventing drug movement in and out of cells (Ho and Kim, 2005). These membrane transporters may be classified as uptake (influx into cell) or efflux (out of cell) transporters, which are typically located either on the basolateral or apical membrane in polarized cells. Interplay of uptake and efflux transporters along with Phase I and II drug metabolizing enzymes may be required for drugs to cross the basolateral and apical membranes of cells (Giacomini et al., 2010).

Transport of drugs across membranes plays an important role in intestinal absorption and first pass extraction of drugs. Drug transport, in this context, involves both passive diffusion and carrier mediated transport [including P-glycoprotein and the Organic anion transporting polypeptide (OATP) family of membrane transporters]. However, transport processes need to be better understood to appropriately interpret the *in vitro* pharmacokinetic transport data in comparison to actual drug absorption and disposition *in vivo*. Another complexity lies in the overlapping substrate specificity of these membrane transporters, such that these substrates tend to be transported by more than one transporter or have drug-transporter interactions (Hagenbuch and Gui, 2008).

Active and facilitative drug transporting proteins, like P-glycoprotein and the OATP family, play an important role in the absorption, distribution, metabolism, and excretion of drugs. P-glycoprotein is highly expressed in the

intestine, liver, brain, and kidneys, and results in decreased absorption in the gut and prevents drugs from crossing the blood-brain barrier (Lin and Yamazaki et al., 2003). Working in opposition to P-glycoprotein are the members of the OATP family of transporters, which act as uptake proteins and facilitate the intestinal absorption of drugs and xenobiotics, as well hepatic uptake and uptake across the blood brain barrier (Hagenbuch and Gui, 2008).

1.4.1: Organic Anion Transporting Polypeptides and Uptake Transport

Organic anion transporting polypeptides (OATP in humans, Oatp in other species) are members of the *SLCO* superfamily of membrane transporters (Hagenbuch and Meier, 2004). They mediate the uptake of a wide number of substrates and have overlapping substrate specificity. The most often studied and best-characterized OATPs are multi-specific and transport substrates in a sodium-independent manner. There are eleven members of the OATP superfamily in humans, classified into six families based on amino acid identity (Hagenbuch and Meier, 2004). OATPs are expressed at the mRNA level in many human tissues, including the liver, blood brain barrier, intestine, choroid plexus, lung, heart, kidney, placenta, and testis (Tamai et al., 2000). Since our interest focuses on of the role of absorption and pre-systemic elimination of fexofenadine, we will focus only on the OATP family members expressed in enterocyte and hepatocyte.

The OATP1 family is the best characterized to date and the largest, with three subfamilies designated OATP1A, 1B, and 1C. OATP1A2 is expressed in many tissues including the apical membrane of the small intestine, blood brain barrier, kidney, and cholangiocytes of the liver (Lee et al., 2005; Glaeser et al., 2007). OATP1B1 and 1B3 are present in the basolateral membrane of the hepatocyte and are thought to be liver specific (König et al., 2000a; König et al., 2000b). The OATP2 family has two members, including OATP2B1 which is expressed in the brain, liver, kidney, lung, and small intestine (Tamai et al., 2001; Kullak-Ublick et al., 2001; St-Pierre et al., 2002; Glaeser et al., 2007). These four OATPs, OATP1A2, 1B1, 1B3, and 2B1, are the focus of this dissertation.

1.4.2: Fexofenadine-Drug and Fexofenadine-Diet Interactions with OATP Family Members

Fexofenadine has been shown *in vitro* to be a substrate for several members of the OATP family of transporters, specifically, OATP1A2 with a K_m of 6 μM (Cvetkovic et al., 1999), OATP1B3 with a K_m of 108 μM (Shimizu et al., 2005), and possibly OATP2B1 (Nozawa et al., 2004). Although these three OATPs appear capable of transporting fexofenadine, their individual contributions to fexofenadine pharmacokinetics are not yet clear. OATP1B1 has also been hypothesized to transport fexofenadine due to its structural similarity to OATP1B3; however, conflicting reports still exist as to whether OATP1B1 is capable of fexofenadine transport (Shimizu et al., 2005; Niemi et al., 2005;

Glaeser et al., 2007; Matsushima et al., 2008). Glaeser et al. (2007) reported that OATP1A2 is the predominant uptake transporter of fexofenadine in the human enterocyte based on the results from both *in vitro* uptake experiments, as well as *in vivo* studies with human subjects.

Some OATPs also transport angiotensin II receptor antagonists, more specifically, valsartan, olmesartan and telmisartan. OATP1B1 and OATP1B3 transport valsartan, with a K_m of 1.4 μM and 18 μM respectively (Yamashiro et al., 2006). Both OATP1B1 and OATP1B3 transport olmesartan in OATP1B1 and OATP1B3 expressing HEK293 cells with K_m values of 12.8 μM and 44.2 μM, respectively (Yamada et al., 2007). OATP1B3 and OATP2B1 are also capable of transporting telmisartan acyl glucuronide (K_m 3.4 μM and 124 μM, respectively), however, OATP1B1 does not transport telmisartan aglucuronide (Ishiguro et al., 2008). Due to the structural similarity between these angiotensin II receptor antagonists, the CYP2C9 probe, losartan, may be a substrate of OATP1A2, OATP1B1, OATP1B3 and/or OATP2B1 and thus lead to drug-drug interactions with fexofenadine at these transporters.

The discovery that grapefruit juice can inhibit fexofenadine transport was one of the earliest reports of a drug-food interaction at the level of transporters (Dresser et al., 2002). The effect of grapefruit juice on drug metabolism was first reported with the calcium channel blockers felodipine and nifedipine (Bailey et al., 1991). Subsequent studies showed that the target of grapefruit juice was not

the liver, but the small intestine, and grapefruit juice caused inhibition of CYP3A4 activity in enterocytes (Lown et al., 1997). Since more than 50% of all drugs are substrates of CYP3A4, the concomitant consumption of grapefruit juice with medications has led to elevated levels of many drugs, drug-drug interactions and even associated drug toxicities. There is increasing evidence that grapefruit juice, as well as other juices also interact with membrane transporters, including the aforementioned P-glycoprotein as well as the OATP family of uptake transporters.

Dresser et al. (2002) measured fexofenadine uptake via OATP1A2 in a transiently transfected HeLa cell line as well as *in vivo* using human subjects. OATP1A2-expressing cells or subjects were given a dose of fexofenadine with water, 25% strength grapefruit juice, normal strength grapefruit, orange, or apple juices or select grapefruit juice extracts. The regular strength juices all inhibited fexofenadine uptake by OATP1A2 in a concentration dependent manner *in vitro.* These juices also reduced the plasma AUC and C_{max} of fexofenadine 30-40% as compared to water alone *in vivo*. The $t_{1/2}$ and t_{max} of fexofenadine were not different among subject treatment groups. Use of a 25% strength grapefruit juice produced a fexofenadine AUC and C_{max} that was decreased 20% compared to that of water (Dresser et al., 2002).

In another study, Dresser et al. (2005) examined the effect of volume of grapefruit juice on the bioavailability of fexofenadine. Using both 300 mL of

grapefruit juice (a volume found in most single serve bottles of grapefruit juice) and 1200 mL of grapefruit juice in combination with an oral dose of fexofenadine, grapefruit juice co-administration decreased the bioavailability of oral fexofenadine significantly. The 300 mL volume of grapefruit juice inhibited fexofenadine adequately to be clinically relevant and was hypothesized to be due to direct inhibition of OATP1A2 (Dresser et al. 2005).

Since inhibition of fexofenadine uptake due to grapefruit juice ingestion represented direct inhibition of OATP1A2, Glaeser et al. (2007) assessed the expression of various human transporters in the intestine, as well as the subcellular localization of OATP1A2, and the effect of grapefruit juice consumption on OATP1A2 expression. Human duodenal biopsy samples were utilized to measure expression of various uptake and efflux transporters *in vivo*. Uptake transporters found at the mRNA level in the small intestine of subjects sampled included OATP1B1, OATP1B3, OATP1A2, and OATP2B1; however, at the protein level, only OATP1A2 and 2B1 were detected. *In vitro* experiments using transiently transfected cell lines showed only OATP1A2 (Cvetkovic et al., 1999; Glaeser et al., 2007) and OATP1B3 (Shimizu et al., 2005) capable of fexofenadine transport. Immunohistochemical staining of human duodenal biopsies showed co-localization of OATP1A2 and P-glycoprotein to the apical brush border of enterocytes (Glaeser et al., 2007). Consumption of grapefruit juice by human subjects two hours before or concomitantly with fexofenadine administration was associated with reduced oral fexofenadine AUC and C_{max},

though intestinal expression of OATP1A2 and P-glycoprotein remained unchanged. In conclusion, Glaeser et al. (2007) hypothesized that OATP1A2 is the key intestinal uptake transporter for fexofenadine and its inhibition by grapefruit juce is responsible for the interaction.

Bailey et al. (2007) further illustrated that flavonoids found in grapefruit juice (naringin) and orange juice (hesperidin) are involved in the direct inhibition of OATP1A2 *in vitro* and decrease the bioavailability of fexofenadine *in vivo*. Naringin had an IC_{50} that was several hundred-fold lower than that normally found in grapefruit juice (3.6 μM) and caused complete inhibition of *in vitro* OATP1A2-mediated fexofenadine uptake. Hesperidin also had a low IC_{50} compared with the typical concentration in orange juice but produced only 60% maximum inhibition. From these studies, naringin and hesperidin seemed to be the main components in their respective juices regarding *in vitro* inhibition of OATP1A2-mediated fexofenadine uptake.

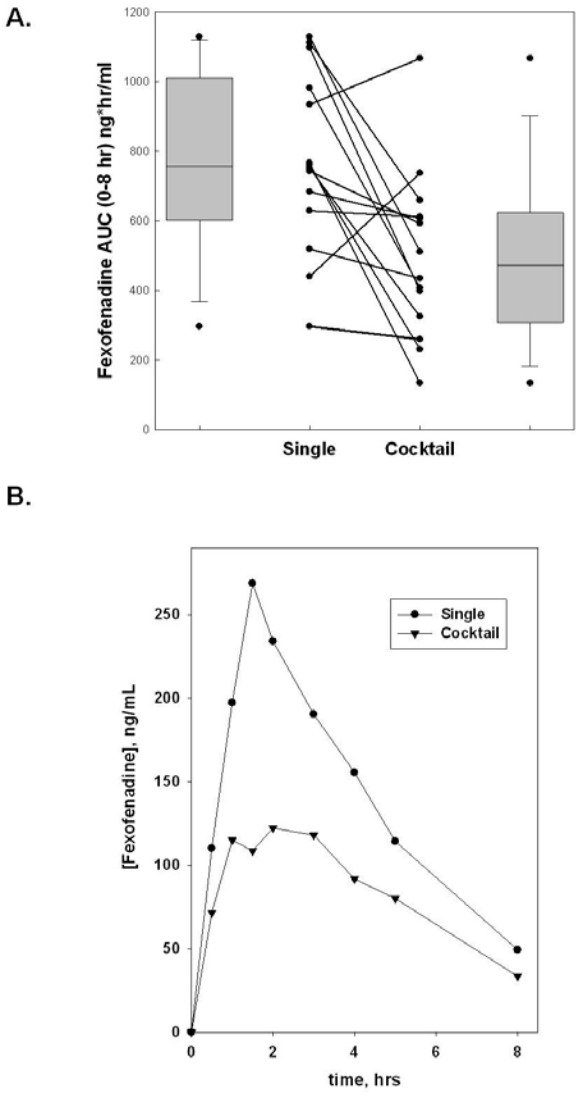
Qiang et al. (2009) reported a fexofenadine-drug interaction in rats involving fluvastatin. Dosing rats orally with the combination of fexofenadine and fluvastatin decreased fexofenadine AUC by 17-50%. However the concurrent use of fluvastatin did not affect the intravenous pharmacokinetics of fexofenadine suggesting that the interaction occurs at the level of the gastrointestinal tract. They hypothesize that the interaction seen between fexofenadine and fluvastatin

may be occurring at an OATP contained within the gastrointestinal tract, sinceboth drugs are substrates of multiple OATPs.

In order to test the Kansas Cocktail in healthy volunteers, initial validation of the probe cocktail was required. Of primary importance was testing for pharmacokinetic interactions between the various probe drugs. To assess whether the probe drugs were interacting, changes in parent/metabolite ratios or changes in AUC were examined. While none of the CYP probes interacted with each other, there was a significant interaction between fexofenadine and one or more of the other probe cocktail components.

When fexofenadine AUC was compared in 14 subjects after administration of fexofenadine alone versus co-administration of fexofenadine with the other four probe compounds as part of the 5-drug cocktail, half of the subjects showed a 50% or greater decrease in the AUC for fexofenadine in the presence of the cocktail (Figure 1.2). Based on this observation, we hypothesized that an enterocyte expressed OATP that was involved in the absorption of fexofenadine was inhibited by one or several CYP probe drugs in the cocktail, resulting in this decrease in AUC.

Figure 1.2:



A Unique Drug-Fexofenadine Interaction Seen With The Kansas Cocktail. A probe drug-fexofenadine interaction was seen in human subjects that decreased fexofenadine AUC by 40-60% in greater than half the subjects (A), as well as decreased C_{max} (B), indicating that this interaction was occurring pre-systemically. Fexofenadine half-life and clearance were not affected.

1.4.3: OATP1A2 Polymorphisms in Humans

The broad substrate specificity of OATP1A2 includes many drugs used clinically and the expression of OATP1A2 is found in organs responsible for drug distribution. Therefore, genetic variations in OATP1A2 (gene *SLCO1A2*) may have significant implications on the disposition of such drugs. Genetic variants may exhibit altered kinetics for transport of a substrate, or may also differ in the occurrence and magnitude of drug-drug or drug-diet interactions.

Lee et al. (2005) identified 6 non-synonymous polymorphisms within the coding region of *SLCO1A2* (Ile13Thr, Asn128Tyr, Asn135Ile, Glu172Asp, Ala187Thr, and Thr668Ser). *In vitro* functional studies revealed that the Glu172Asp and Asn135Ile variants had reduced uptake capacity of model substrate estrone-3-sulfate and two opioid receptor antagonists. Other variants had altered transport activity that was substrate specific. Further kinetic analysis of transport data indicated that mutations at codons 128, 135, 172, 187, and 668 would have functional consequences (Lee et al., 2005).

Badagnani et al. (2006) identified seven novel OATP1A2 variants from 270 ethnically diverse samples. Four of the seven variants were found to be protein altering and subsequently had altered transport of estrone-3-sulfate and/or methotrexate. Single nucleotide polymorphism Ile13Thr increased model substrate transport creating a hyperfunctional OATP1A2, variant Arg168Cys

created a hypofunctional OATP1A2, and the Glu172Asp variant created an OATP1A2 with reduced maximal transport capacities.

1.5: Specific Aims of This Dissertation

Membrane transporters are key modulators, altering disposition of drugs and other chemicals, via absorption, distribution, metabolism, and elimination from the body. While the importance of membrane transporters is well understood, better-characterized tools are needed to support continuing investigations of their specific functions both *in vitro* and *in vivo*. To better study and understand transporter function *in vivo* a safe and well-characterized probe drug is required. Fexofenadine is a promising probe for the study of transporter functions in humans because the drug is incredibly safe, is essentially not metabolized, and a substrate for transporters. Use of fexofenadine as a probe, however, requires a clear understanding of the roles of the different transporters involved in its disposition. The long-term goal of this dissertation is to define the utility of fexofenadine as a probe for characterizing the activity of uptake and efflux transporters in humans. By doing so, we can identify sources of within and between-subject variability (e.g., transport polymorphisms), and to predict drug-drug interactions. Ultimately, this research will contribute the optimization of drug therapy.

Several members of the organic anion transporting polypeptide (OATP) family of transporters are thought to contribute to fexofenadine pharmacokinetics; however their individual contributions have not been well defined. Since there

are conflicting reports as to the contribution of specific transporters on the pharmacokinetics of fexofenadine, the objective of this dissertation was to identify the dominant transporter(s) involved in fexofenadine pharmacokinetics and to further validate the utility of fexofenadine as a probe for transporter activity in humans. The previously unreported fexofenadine-drug interaction in a clinical study provides an additional approach for defining the specific transporter(s) of fexofenadine uptake and efflux.

To understand the role(s) of transporters in drug-drug interactions, defining the dominant transporter(s) of fexofenadine and their relative contributions will illustrate best how human transporters function and how this function can be modulated. The central hypothesis of this study is that drug-drug interactions at a transporter facilitating uptake of fexofenadine decreases fexofenadine uptake *in vivo*, and thereby alters fexofenadine pharmacokinetics. We tested this hypothesis with the following specific aims:

1.5.1: Specific Aim 1

Development and validation of an LC-MS/MS method for the measurement of fexofenadine. The method developed was specific for fexofenadine, rapid, and with sufficient sensitivity for the studies proposed.

Based on previous reports using radiolabeled fexofenadine quantities of fexofenadine taken up by transporters are in the picogram range.

1.5.2: Specific Aim 2

First, it was important to define and characterize the kinetics of fexofenadine with human OATPs expressed in the enterocyte and the hepatocyte. The transporters studied were OATP1A2, 1B1, 1B3, and 2B1. Using transiently transfected HEK293 cells expressing OATP1A2, as well as stable CHO cell lines for OATP1B1, 1B3, and 2B1, fexofenadine kinetics (K_m) were measured. The analytical method developed in Specific Aim 1 was used to measure fexofenadine uptake in these systems. The studies proposed test the current hypothesis that OATP1A2 is capable of fexofenadine transporter in the human enterocyte and hepatocyte.

1.5.3: Specific Aim 3

Identification of probe drug(s) interacting with fexofenadine at previously described transporter(s) *in vitro* allowed the determination of fexofenadine-drug interaction seen clinically. Based on observations in a previous clinical study, a decrease in fexofenadine C_{max} and AUC occured when fexofenadine was dosed

as part of a 5-drug cocktail (losartan, buspirone, fexofenadine, caffeine, and dextromethorphan) as compared to a single dose of fexofenadine. Characterization of the interaction of probe drugs with fexofenadine at a specific transporter(s) provides further support for the identity of the dominant uptake transporter controlling fexofenadine pharmacokinetics. To examine drug-drug interactions with membrane transporters, we used the same transiently transfected HEK293 cells and stably transfected CHO cell lines described in Specific Aim 2. Fexofenadine transport was measured by LC-MS/MS and the effects of the other four probe drugs were determined.

1.5.4: Specific Aim 4

Exploration of the effect of OATP1A2 genotypes on fexofenadine transport kinetics and fexofenadine-probe drug interactions was also studied. Using three known human single nucleotide polymorphisms (SNPs), we used site-directed mutagenesis to create three OATP1A2-mutant transporters. After verification of their sequences, we transiently transfected HEK293 cells with each of the mutated transporters and determined the effects of these mutations on the transport of fexofenadine as well as the effects of the other probe drugs on fexofenadine transport.

Chapter 2

Experimental Materials and Methods

2.1: Materials

[^{3}H] Estrone-3-sulfate (E3S; 54.26 Ci/mmol) and [^{3}H] estradiol-17β-glucuronide (E17-βG; 41.8 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Sodium butyrate was purchased from Sigma-Aldrich (St. Louis, MO). Fexofenadine hydrochloride was obtained from Toronto Research Chemicals (Toronto, Ontario, Canada), buspirone hydrochloride, caffeine, dextromethorphan hydrobromide, losartan potassium, and cetirizine hydrochloride (internal standard, IS) were obtained from Sigma Aldrich (St. Louis, MO, USA). Ammonium formate, methanol, acetonitrile, and formic acid, all of HPLC or Optima grade, were from Fisher Scientific (Fair Lawn, NJ, USA). Purified water was from a Millipore Q Water System (Millipore, Bedford, MA, USA). All other chemicals were analytical grade and purchased from Sigma-Aldrich or Fisher Scientific.

Human embryonic kidney (HEK293) cells, Eagle's minimum essential medium, and Dulbecco's modified Eagle's medium (high glucose) were from American Type Culture Collection (Manassas, VA). Dulbecco's modified Eagle's medium (low glucose) was purchased from Caisson Laboratories (North Logan, UT). Fetal bovine serum

(FBS) was obtained from HyClone (Logan, UT). Sulfo-NHS-SS-biotin, NeutrAvidin agarose beads and the BCA protein assay kit were from Thermo Fisher Scientific (Rockford, IL). All other cell culture materials were purchased from Sigma-Aldrich or Invitrogen (Carlsbad, CA).

2.2: LC-MS/MS Sample Preparation

Fexofenadine and cetirizine (IS) stock solutions (1 mg/mL) were individually prepared in methanol. Busprione, caffeine, dextromethorphan, and losartan stock solutions (1 mg/mL) were individually prepared in water. The internal standard was diluted to 100 ng/mL (working concentration) by diluting the stock solution with and a diluent composed of 7.5 mM ammonium formate, pH 5, methanol, acetonitrile (50:25:25, v/v/v). This same diluent was used for all dilutions and for sample reconstitution.

HEK293 cell culture lysates were spiked with 25 or 50 µL of fexofenadine working solutions to obtain final fexofenadine concentrations of 0, 1, 2, 5, 10, 50, 100, and 500 ng/mL fexofenadine, containing the IS at a concentration of 10 ng/mL. Quality control (QC) samples were prepared independently on separate days at concentrations of 3 (low), 75 (medium), 400 and 500 (high) ng/mL fexofenadine. Similar working solutions were also made for the other components of the probe drug cocktail (buspirone, caffeine, dextromethorphan, and losartan). HEK293 cell culture lysates were spiked with probe drugs to obtain final concentrations of of 0-500 ng/mL probe

drug, with the IS at a concentration of 10 ng/mL. QC samples were prepared independently for each probe drug at concentrations between 3 and 500 ng/mL.

For *in vitro* studies of fexofenadine transport, the matrix was a mammalian cell lysate derived from HEK293 cells transiently transfected with the uptake transporter OATP1A2. Confluent monolayers of HEK293 cells containing OATP1A2 in 24-well plates were washed with uptake buffer (142 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM glucose and 12.5 mM Hepes, and was adjusted to pH 7.4 with Tris base) three times and then frozen overnight. Cells then were thawed and 150 µL of diluent containing the desired concentration of fexofenadine and the internal standard (final concentration 10 ng/mL cetirizine) was added and cells were lysed at room temperature for 20 minutes on a rocker platform. This mixture was centrifuged at 20,000 rpm for 5 minutes to pellet precipitated protein. Lysates were then transferred to a round bottom 96-well plate and 10 µL was injected for analysis.

2.3: LC-MS/MS Conditions

Chromatography was performed using a Luna C18 column (5 µm, 50 x 2 mm), fitted with a C18 4 x 2 mm guard column (Phenomenex, Torrance, CA, USA) at 40 ºC. The aqueous mobile phase (Solvent A) was 7.5 mM ammonium formate, pH 5, and the organic mobile phase (Solvent B) was acetonitrile and methanol (50:50, v/v). Total flow rate was 0.5 mL/min. The gradient used allows the quantification not only of fexofenadine but also of the other probe drugs employed, namely buspirone, caffeine, dextromethorphan, and losartan. Elution was performed with a linear gradient from 5%

to 90% Solvent B from 0-2 minutes, elution with 90% B for 0.5 minutes, and then a linear decrease to 5% B over 0.75 minutes. Total run time was 5 minutes.

Mass spectrometry was performed using a Waters Quattro Premier mass spectrometer (Waters Corporation, Milford, MA, USA) in the positive ion electrospray mode. Source temperature was 120 ºC, with a desolvation temperature of 350 ºC. Cone gas flow was set at 60 L/hr and a desolvation gas flow of 650 L/hr. The mass spectrometer was operated in the MRM mode with a dwell time of 0.050 seconds per MRM channel. Fexofenadine, probe drugs, or internal standard (cetirizine) at a concentration of 100 µg/mL in methanol was infused directly into the source of the mass spectrometer to determine characteristic product ions of the analytes and IS (Table 2.1). Data acquisition was performed with MassLynx (Version 4.12) and quantitation of results with QuanLynx software.

Table 2.1: Characteristic Product Ions of The Kansas Cocktail and Internal Standard.

Analyte	Precursor Ion (m/z)	Product Ion (m/z)	Cone (V)	Collision (V)
Fexofenadine	502.17	466.2	44	26
Buspirone	386.32	122.3	50	30
Caffeine	195.26	138.2	35	30
Dextromethorphan	271.28	121.22	40	18
Losartan	422.98	207.2	26	24
Cetirizine (IS)	389.02	201.1	30	20

2.4: Bioanalytical Method Validation

Standards for calibration curves were prepared by spiking aliquots of diluent or cell culture lysate with 10 ng/mL cetirizine (IS) and seven non-zero analyte concentrations, covering a range of 1-500 ng/mL fexofenadine. The calibration curves for standards in diluents and in cell lysates, were generated on consecutive days from samples injected in duplicate using the analyte to IS peak area ratios by weighted (1/x) least squares linear regression. Acceptance criteria for calibration curves were a correlation coefficient (r^2) of 0.99 or greater and standard concentrations determined within 15% of the actual value, the exception being the LLOQ which must be within 20% of the actual value. The precision and accuracy of the method was determined by analyzing three sets of QC samples (low, medium, high), with each batch containing five replicates of each concentration level, analyzed on consecutive days. Precision of the method was calculated by determining the coefficient of variation (CV). The acceptable criteria for intra- and inter-day precision were <15% for all non-LLOQ samples and <20% for the LLOQ. Accuracy of the method was considered acceptable if measured values were between 85-115% of the actual value, or 80-120% for the LLOQ.

Extraction recovery of the analyte (fexofenadine) was determined by comparing the raw peak area of fexofenadine isolated from spiked cell culture matrix to the peak area of the analyte at the same concentration in diluent. Similar methodology was used

to validate the other 4 components of the probe drug cocktail (buspirone, caffeine, dextromethorphan, and losartan) and met the same criteria for method validation.

2.5: Cell Culture

HEK293 cells were grown in Dulbecco's modified Eagle's medium containing 4.5 g/L D-glucose, 2 mM L-glutamine, 25 mM Hepes buffer, and 110 mg/L sodium pyruvate, supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. CHO cells stably expressing OATP2B1 or the empty vector were grown in F-12 Nutrient Mixture containing 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 400 µg/mL Hygromycin B. CHO cells stably expressing OATP1B1, OATP1B3, or wild-type (WT) were grown in Dulbecco's modified Eagle medium, containing 1 g/L D-glucose, 2 mM L-glutamine, 25 mM Hepes buffer and 110 mg/L sodium pyruvate, supplemented with 10% FBS, 50 µg/mL L-proline, 100 U/mL penicillin and 100 µg/mL streptomycin. All cells were maintained in a humidified environment at 37°C and 5% CO_2.

2.6: OATP Expression

HEK293 cells (200,000 cells per well) were seeded on 24-well plates pre-treated with poly-D-lysine, and were transfected with pExpress-1 (Express Genomics, Inc., Frederick, MD) containing OATP1A2, or the empty vector, and Fugene HD (Roche Diagnostics, Indianapolis, IN) 48 hours prior to uptake experiments. CHO cells stably transfected with human OATP2B1 have been previously described (Pacyniak et al., 2010). CHO cells stably expressing OATP2B1 or the empty vector (pCDNA5/FRT) were seeded on 24-well plates (100,000 cells per well) 48 hours prior to uptake experiments; OATP2B1 expression in this cell line did not require sodium butyrate induction. OATP1A2 mutant constructs were also transiently expressed in HEK293 cells. Cells were seeded in 24-well tissue culture plates (200,000 cells per well), pre-coated with poly-D-lysine. At 90% confluence, cells were transfected with wild-type or mutant OATP1A2 constructs (Badagnani et al., 2006) or the empty plasmid using FuGENE-HD transfection reagent (Roche, Basel, Switzerland), following manufacturer's instructions. All experiments were performed forty-eight hours after transfection. CHO cells stably transfected with human OATP1B1 and OATP1B3 have been previously described (Gui et al., 2008). CHO cells stably expressing OATP1B1 or OATP1B3 or wild-type cells were plated at 40,000 cells per well on 24-well plates and 48 hours later medium was replaced with medium containing 5 mM sodium butyrate to induce nonspecific gene expression. After an additional 24 hours in culture, the cells were ready for uptake experiments.

2.7: Transport Experiments

For experiments with OATP1A2, HEK293 uptake buffer contained 142 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM glucose and 12.5 mM Hepes, and was adjusted to pH 7.4 with Tris base. For experiments with OATP1B1, 1B3, and 2B1, CHO uptake buffer contained 116.4 mM NaCl, 5.3 mM KCl, 1 mM NaH_2PO_4, 0.8 mM $MgSO_4$, 5.5 mM D-glucose and 20 mM HEPES, and was adjusted to pH 7.4 with Tris base. For experiments using the OATP1A2 mutants, HEK293 buffer was used.

Cells in 24-well plates were washed with uptake buffer pre-warmed to 37°C and then incubated on a 37°C warming block with uptake buffer containing substrates and/or inhibitors for the indicated time periods. Uptake was terminated by removing the uptake solution and washing the cells with 4°C uptake buffer. To measure uptake of radiolabeled substrates, cells were lysed with 1% Triton X-100 in PBS, and the radioactivity was quantified with liquid scintillation counting. Protein concentrations were determined with a BCA assay kit (Thermo Fisher Scientific, Rockford, IL) and uptakes were normalized based on protein concentration.

Unlabeled probe drugs were measured using high performance liquid chromatography-tandem mass spectrometry (LC-MS/MS). Cells were lysed in a mobile

phase of 50:50 (v/v) 7.5 mM ammonium formate, pH 5 and 50:50 (v/v) acetonitrile and methanol containing 10 ng/mL cetirizine as an internal standard and prepared for analysis as described previously (Flynn et al., 2011). Probe drugs were separated by reverse phase chromatography using a Phenomenex C18 column (Phenomenex Inc., Torrance, CA, 50 x 2.1mm, 5 µm) at 40 °C. Mobile phase consisted of 7.5 mM ammonium formate, pH 5 and 50:50 (v/v) acetonitrile and methanol. The flow rate was 0.5 mL per minute with gradient elution, allowing a run time of 5 minutes. Drugs were detected and quantified in electrospray positive ion mode with a Waters Quattro Premier (Waters Corporation, Milford, MA). The transitions monitored were 502.17 > 466.2 for fexofenadine, 386.32 > 122.3 for buspirone, 195.26 > 138.2 for caffeine, 272.34 > 171 for dextromethorphan, 422.98 > 207.2 for losartan, and 389.1 > 201.1 for cetirizine (internal standard). QuanLynx software (Waters Corporation, Milford, MA) was utilized to quantify mass spectrometry data.

Protein concentrations were determined with a BCA assay kit (Thermo Fisher Scientific, Waltham, MA), and uptake was normalized to protein. Net OATP-mediated uptake was defined as the uptake by OATP-expressing cells minus the uptake by the control cell line (HEK293 cells transiently expressing the empty vector p-Express 1 for transiently expressed OATP1A2 and its mutants, CHO cells stably expressing the empty vector pCDNA5/FRT for OATP2B1, and wild-type CHO cells for OATP1B1 and OATP1B3).

2.8: Surface Biotinylation and Western Blot Analysis

Cell surface expression was measured by incubating cells with 1 mg/ml sulfo-NHS-SS- biotin in PBS for 1 hour at 4°C. After stopping the reaction with 100 mM glycine in phosphate buffered saline, cells were lysed for 10 minutes on ice, in buffer containing 10 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100 and protease inhibitors. This lysate was centrifuged at 10,000 x g for 2 minutes. Cell supernatant was added to 70 µL neutravadin agarose beads and incubated for 1 hour at room temperature. Beads were washed three times for 5 minutes with lysis buffer, then the biotinylated proteins were eluted by incubating with 2x Laemmli buffer containing 5% 2-mercaptoethanol for 30 minutes. Protein was run on a 6% SDS-PAGE gel and transferred to nitrocellulose membrane. Western blots were probed with a monoclonal OATP1A2 antibody from Santa Cruz Biotechnology (Valencia, CA), following the manufacturer's recommendations.

2.9: Data and Statistical Analysis

Transporter-mediated net uptake was obtained by subtracting the uptake in control cells from that in OATP-expressing cells. Inhibition of transporter-mediated uptake was calculated based on the difference between net uptake of a substrate in the absence of inhibitor and net uptake in the presence of inhibitor. Data were fit to

Michaelis-Menten kinetics using GraphPad Prism 5 (La Jolla, CA). Statistical analysis of differences between treatments was performed using SigmaPlot 11.0 utilizing a one-way analysis of variance (one-way ANOVA) and Bonferoni post-hoc test (SyStat Software Inc., San Jose, CA). P-values less than 0.05 ($p<0.05$) are considered significant and were denoted by an asterisk.

Chapter 3

Quantification of the Transporter Substrate Fexofenadine in Cell Lysates by LC-MS/MS

3.1: Abstract

Drug-drug interactions at transporters present a significant and under-investigated clinical problem. Investigations of specific transporter functions and screening for potential drug-drug interactions, both *in vitro* and especially *in vivo,* will require validated experimental probes. Fexofenadine, an approved, well-tolerated drug, is a promising probe for studies of membrane transporter function. Although fexofenadine pharmacokinetics are known to be controlled by transporters, the contributions of individual transporters have not been defined. We have developed a rapid, specific, and sensitive analytical method for quantitation of fexofenadine to support this work. This LC-MS/MS method quantifies fexofenadine in cell lysates from *in vitro* studies using cetirizine as the internal standard. Cell lysates were prepared for analysis by acetonitrile precipitation. Analytes were then separated by gradient reverse-phase chromatography and analyzed by tandem mass spectrometry using the *m/z*

 Rapid Commun. Mass Spectrom. 2011, 25, 2361–2366.

502.17/466.2 transition for fexofenadine and *m/z* 389.02/201.1 for cetirizine. The method exhibited a linear dynamic range of 1–500 ng/mL for fexofenadine in cell lysates. The lower limit of quantification was 1 ng/mL with a relative standard deviation of less than 5%. Intra- and inter-day precision and accuracy were within the limits presented in the FDA guidelines for bioanalysis. We also will validate this method to support not only the quantification of fexofenadine, but also other probe drugs for drug-drug interaction studies. This method for quantification will facilitate the use of fexofenadine as a probe drug for characterization of transporter activity.

3.2: Introduction

Fexofenadine, a non-sedating H_1-receptor antagonist, is a substrate of the uptake transporter organic anion transporting polypeptide 1A2 (OATP1A2) and the efflux transporter, P-glycoprotein (P-gp) (Cvetkovic et al., 1999; Glaeser et al., 2007). Additional reports have suggested that OATP1B1, OATP1B3, and OATP2B1 might also transport fexofenadine (Kim 2003; Shimizu et al., 2005). Ninety-five percent of fexofenadine is excreted unchanged, with only 1% metabolized prior to excretion (Lippert et al., 2005), thus fexofenadine pharmacokinetics are controlled by transporters, rather than by metabolism. The pharmacokinetics of fexofenadine suggests that it might be a useful probe drug for functional studies of both the OATP family of transporters and P-glycoprotein (Dresser et al.; 2002). However, the precise contributions of individual transporters to fexofenadine pharmacokinetics are still

unknown (Hamman et al., 2001; Dresser et al., 2002; Wang et al., 2002). This will require careful characterization of fexofenadine transport in model systems to define the relative roles of these various transporters. Both *in vitro* studies to identify transporters with high affinity for fexofenadine, and *in vivo* studies to characterize the role of fexofenadine as a selective probe for certain transporter activity, will require a bioanalytical method that is accurate, rapid, sensitive, and selective for this probe drug.

Previous methods for quantification of fexofenadine have been described based on HPLC using fluorescence detection (Coutant et al., 1991; Uno et al., 2004). Those methods, however, required relatively long analysis times and provide less sensitivity and specificity than required for our studies. Due to these issues, high selectivity and sensitivity LC-MS/MS methods were developed for the quantification of fexofenadine. However, these methods had relatively long run times (>10 minutes) and were validated for the quantification of fexofenadine in plasma or urine samples (Hofmann et al., 2002; Fu et al., 2004). To meet the need for the bioanalytical support for cell-based transporter assays, we have developed and validated an LC-MS/MS method for the identification and quantification of this drug in cell culture lysates using cetirizine as the internal standard. This method will be applied to the analysis of fexofenadine in mammalian cell lysates from *in vitro* transporter studies, and will be developed further to measure other probe drugs to support drug-drug interaction studies in these model systems.

3.3: Results

3.3.1: Mass Spectrometry.

Figure 3.1.1 and 3.1.2 shows the mass spectra obtained for fexofenadine and the internal standard cetirizine. Fragmentation of fexofenadine shows its characteristic product ions at m/z of 131.1, 171.2, 189.0, 233.9, 262.3, and 466.2. The two most abundant ions are 466.2 and 171.2 (m/z). For cetirizine a characteristic product ion at m/z 200.9 was observed. The most sensitive transitions were 502.17/466.2 for fexofenadine and 389.09/201.1 for the internal standard cetirizine.

3.3.2: Chromatography.

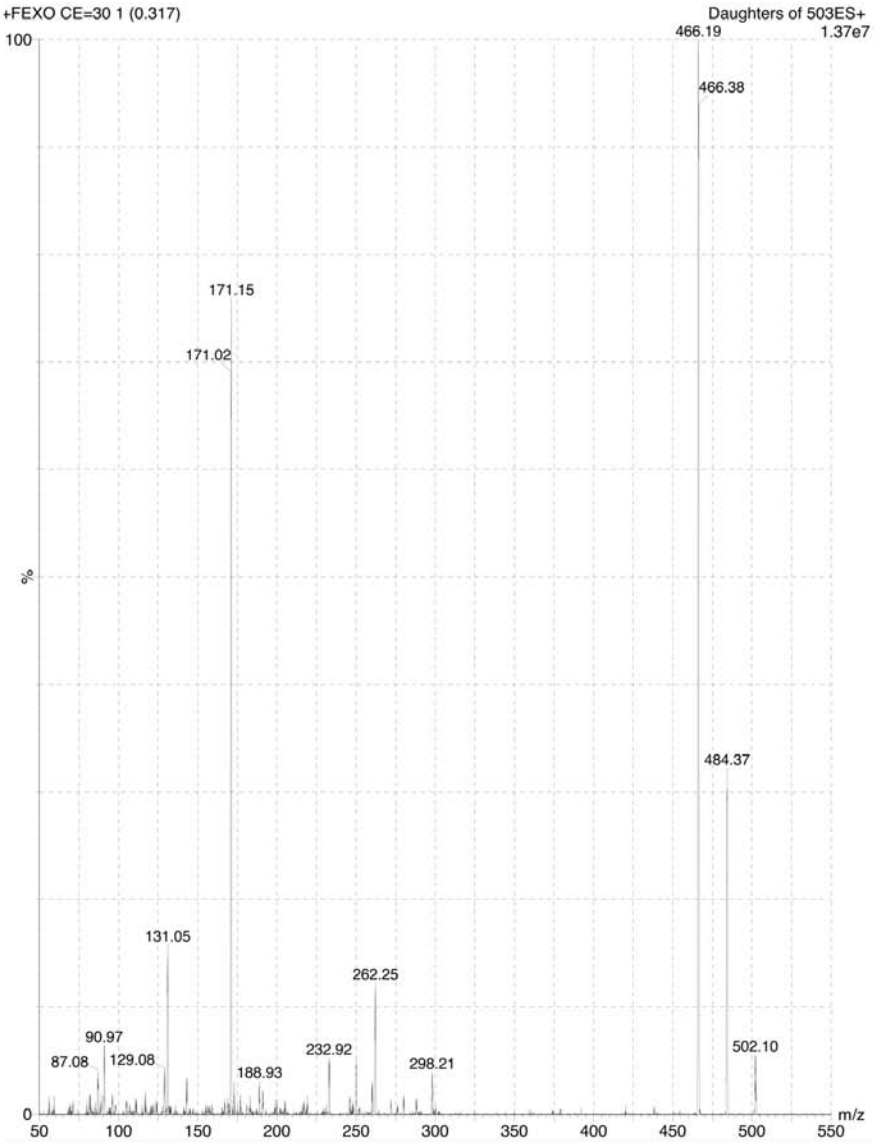
Chromatographic conditions were optimized by testing aqueous mobile phases that varied in pH from 3 to 8, and by using methanol, acetonitrile, or a 50/50 (v/v) methanol-acetonitrile mixture as the organic phase. Based on sensitivity, peak shape for the analyte and internal standard, and on overall run time, we found that a gradient of ammonium formate (7.5 mM, pH 5) and acetonitrile-methanol (50:50, v/v) achieved the best chromatographic results. This mobile phase allowed elution of fexofenadine at 2.48 minutes and the internal standard at 2.66 minutes with a total run time of less than five minutes. Although ideal for analysis of fexofenadine alone, we also anticipate the

need to measure additional analytes as we investigate drug-drug interactions at membrane transporters. To support this future need, we modified the method to include other probe drugs of interest. Use of the same gradient we developed for fexofenadine alone also yielded acceptable retention of the relatively polar probe drug caffeine and the elution of fexofenadine, three other less polar probe drugs (buspirone, dextromethorphan, losartan), and the internal standard within 5 minutes (data not shown).

3.3.3: Selectivity.

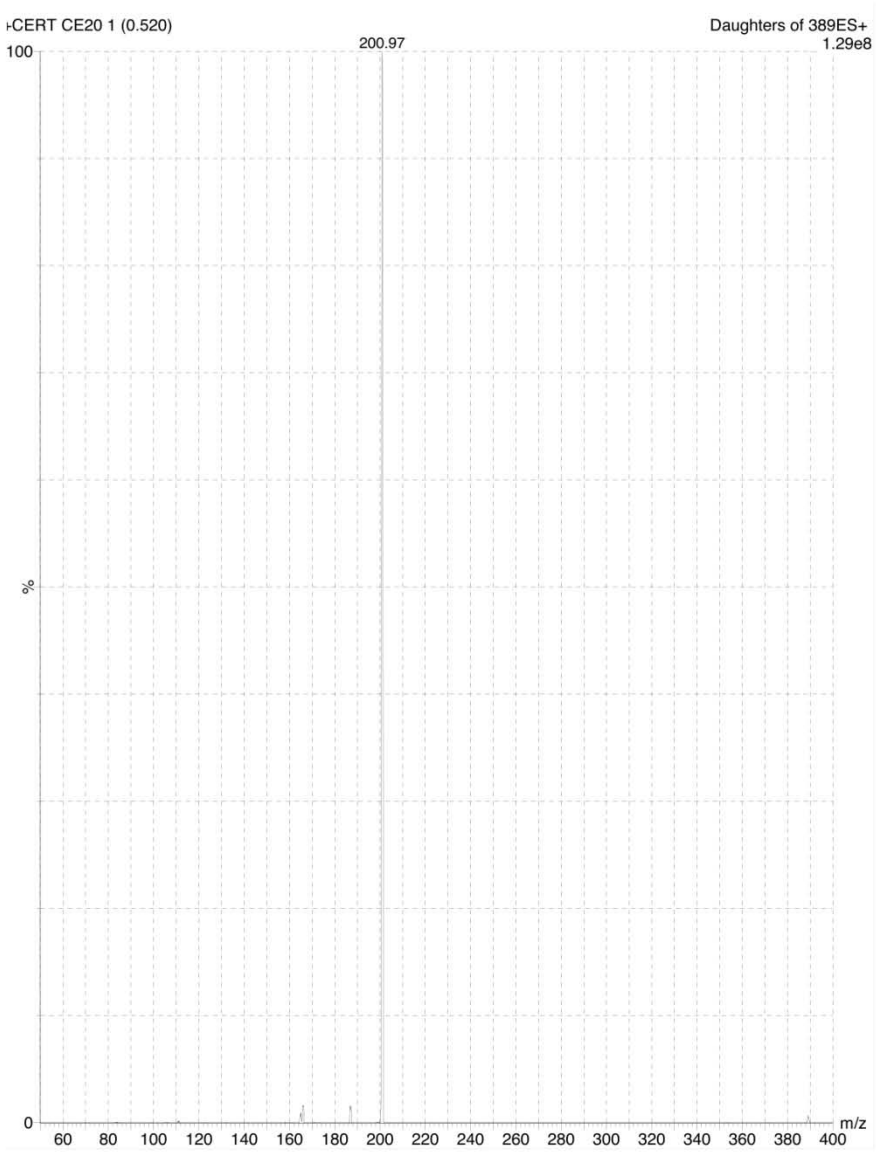
Method selectivity was examined by analyzing blank cell culture matrix samples and matrix spiked only with IS. As seen in Figure 3.2.1, there is no endogenous compound interference at the retention times of the analyte or internal standard. Similarly, the internal standard does not show any crossover in the MRM of the analyte in Figure 3.2.2. The MRM chromatogram in Figure 3.2.3 illustrates the result from a sample spiked with 1 ng/mL fexofenadine, demonstrating the sensitivity of the method.

Figure 3.1.1:



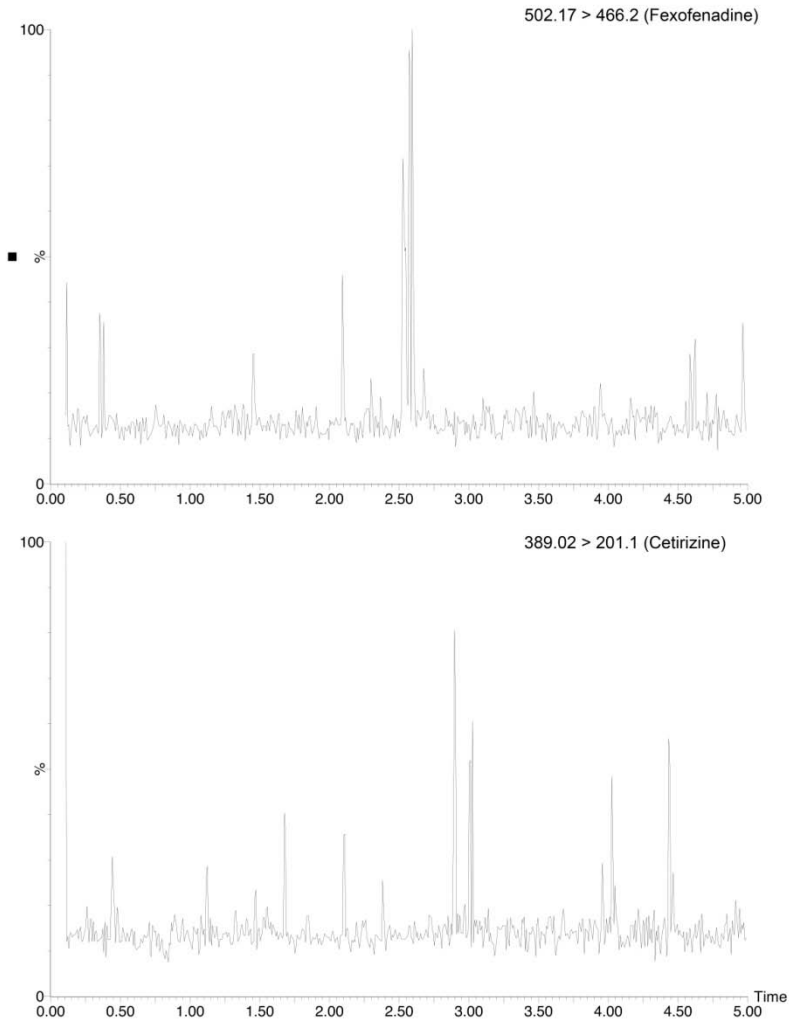
Mass Spectrum Obtained For Fexofenadine. A product spectrum for fexofenadine was generated by direct infusion into the ESI probe with the most sensitive transition being 502.17/466.2 m/z.

Figure 3.1.2:



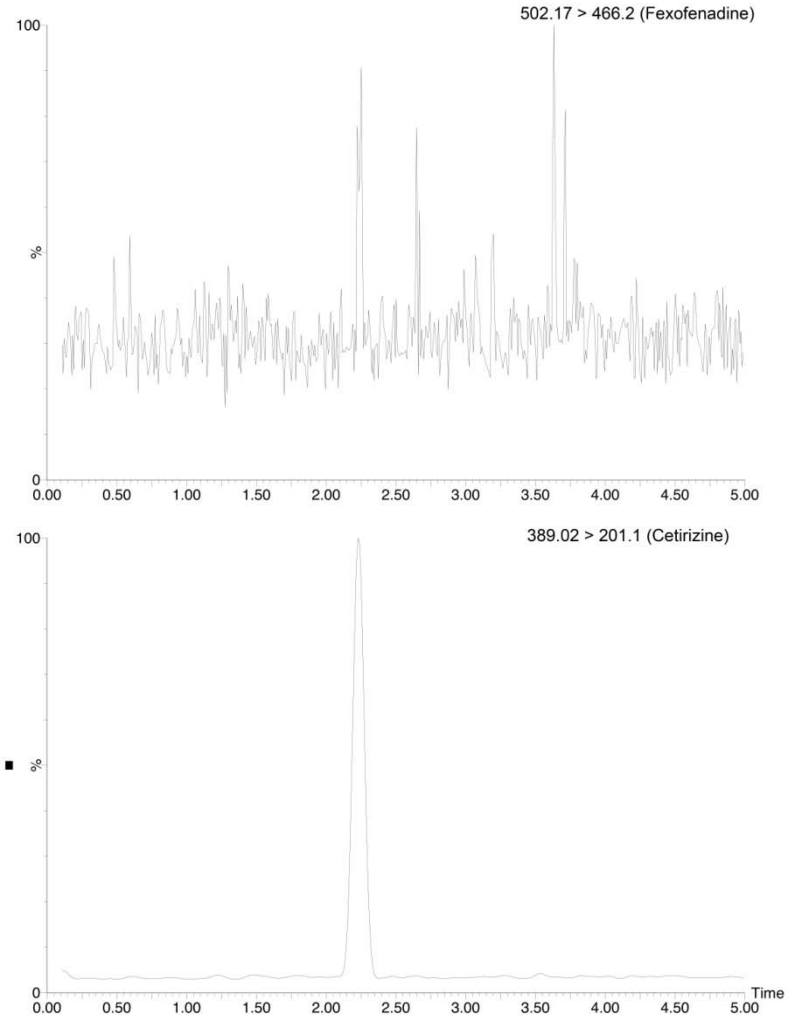
Mass Spectrum Obtained For Cetirizine. A product spectrum for cetirizine was generated by direct infusion into the ESI probe with the most sensitive transition being 389.09/201.1 m/z.

Figure 3.2.1:



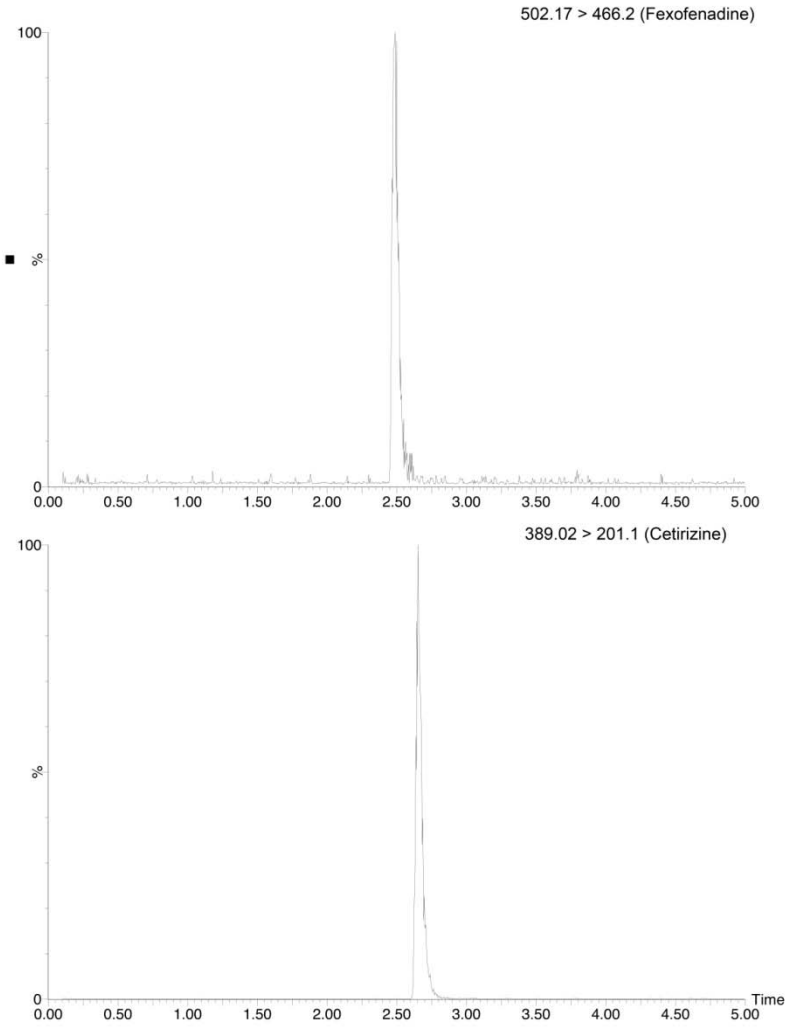
MRM Chromatogram From Blank Cell Lysate. Aliquots of HEK293 cell lysates were spiked, prepared by lysis with mobile phase, analyzed by LC-MS/MS, and quantified as described in Materials and Methods. This chromatogram is from analysis of a sample prepared from blank cell lysate only.

Figure 3.2.2:



MRM Chromatogram of Cetirizine (Internal Standard). Aliquots of HEK293 cell lysates were spiked with cetirizine, prepared by lysis with mobile phase, analyzed by LC-MS/MS, and quantified as described in Materials and Methods. This chromatogram is from analysis of a sample that contained 10 ng/mL cetirizine only.

Figure 3.2.3:

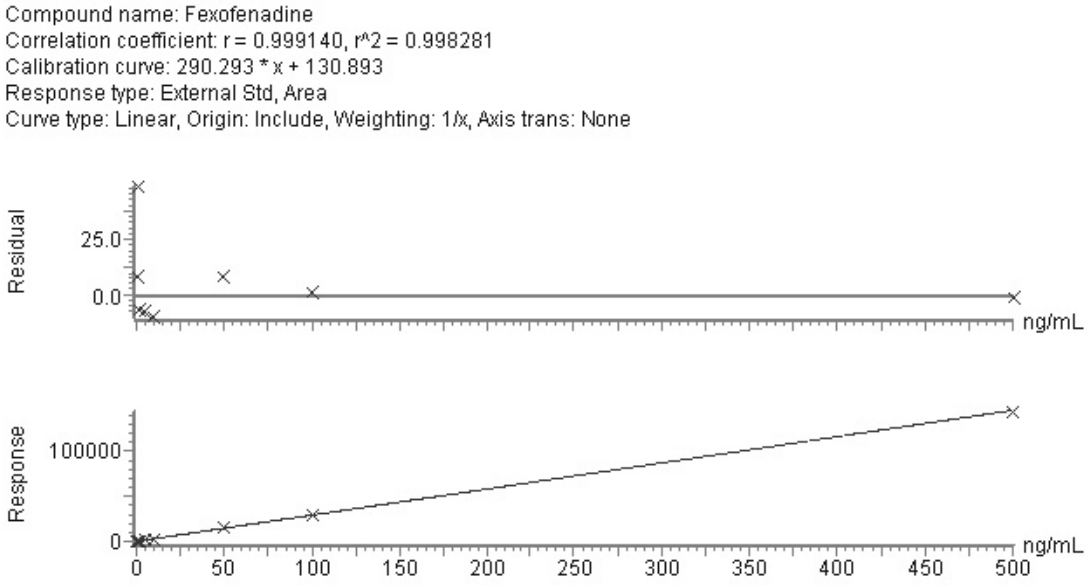


MRM Chromatogram of Fexofenadine and Cetirizine. Aliquots of HEK293 cell lysates were spiked with cetirizine and fexofenadine, prepared by lysis with mobile phase, analyzed by LC-MS/MS, and quantified as described in Materials and Methods. This chromatogram is from analysis of a sample containing both 10 ng/mL internal standard and fexofenadine at the LLOQ, 1 ng/mL.

3.3.4: Linearity.

The seven-point calibration curve for fexofenadine extracted from HEK293 cell lysates was linear over the selected concentration range of 1-500 ng/mL fexofenadine. The best linear fit and linear regression were obtained with a 1/x weighing factor. Figure 3.3 illustrates a representative standard calibration curve for fexofenadine in cell lysates, with an r^2 of 0.99. Table 3.1 summarizes the calibration curve results, illustrating our ability to both precisely and accurately measure fexofenadine in mobile phase. Fexofenadine quantitation at 1 ng/mL was accomplished with a precision (CV) of 2.6% and a mean accuracy of 106.5%, well within the limits for defining the LLOQ. Similarly, the precision and accuracy of each concentration of fexofenadine in the calibration curve met the FDA criteria for bioanalytical method validation (FDA, 2001). The inter-day coefficient of variation (CV) for the lowest QC standard (3 ng/mL) in cell lysate is 9.5% and the between batch accuracy was 97.4% (Table 3.2). Within the batches, the low QC standard had a CV of 8.5% and the accuracy was 91.9%. For the middle and upper quantification levels (ranging from 75-500 ng/mL), the precision (CV) ranged from 2.5-6.2% and the accuracy from 90.1-97.0% within batch (Table 3.2). Between batches, the precision ranged from 3.4-4.9% and the accuracy from 97.4-103.6% in HEK293 cell lysate.

Figure 3.3:



Calibration Curve for Fexofenadine in HEK293 Cell Lysate. Aliquots of HEK293 cell lysates were spiked with the from 0-500 ng/mL fexofenadine and with 10 ng/mL of the internal standard, cetirizine. Samples were prepared by prepared by lysis with mobile phase, analyzed by LC-MS/MS, and quantified as described in Materials and Methods.

Table 3.1: Precision and Accuracy Data for Fexofenadine Calibration Standards From Spiked HEK293 Cell Lysate.

Concentration Added (ng/mL)	Concentration Found (ng/mL)	Precision (CV, %)	Accuracy (%)
1	1.06	2.6	106.5
2	1.98	7.6	98.9
5	4.73	2.1	94.5
10	8.79	3.5	87.9
50	53.5	1.7	106.9
100	99.2	2.5	99.2
500	498.6	0.7	99.7

Table 3.2: Intra- and Inter-Day Precision and Accuracy of the Method For Determining Fexofenadine Concentrations in HEK293 Cell Lysates.

	Intra-day (n = 5)		
Concentration Added (ng/mL)	**Concentration found (mean) (ng/mL)**	**Precision (CV, %)**	**Accuracy (%)**
3	2.76	8.5	91.9
75	72.8	3.5	97.0
400	373.9	6.2	93.5
500	450.6	2.5	90.1

	Inter-day (n = 3)		
Concentration Added (ng/mL)	**Concentration found (mean) (ng/mL)**	**Precision (CV, %)**	**Accuracy (%)**
3	2.92	9.5	97.4
75	76.3	3.4	101.7
400	404.5	4.9	103.6
500	500.8	4.3	100.2

3.3.5: Recovery.

Table 3.3 illustrates the recovery of fexofenadine from HEK293 cell lysate as compared to samples spiked in mobile phase. The average recovery of fexofenadine from spiked cell culture matrix was from 86% to 123%. With the exception of the LLOQ, all concentrations had standard deviations of recovery less than 20%.

3.4: Discussion

Fexofenadine is a promising probe for transporter function both *in vitro* and especially *in vivo*. In order to use fexofenadine as a probe of transporter function and measure fexofenadine transporter activity, a rapid, specific, and sensitive analytical method has been developed to meet this need. Compared to other analytical methods, including HPLC with fluorescence detection (Coutant et al., 1991; Uno et al., 2004) and existing LC-MS/MS methods (Hofmann et al., 2002; Fu et al., 2004; Yamane et al., 2007) we are able to provide improved selectivity and sensitivity. Our method yields a LLOQ of 1 ng/mL, versus previously published LLOQs of greater than 1 ng/mL (Coutant et al., 1991; Dresser et al., 2002; Hofmann et al., 2002; Fu et al., 2004; Yamane et al. 2007). While the LLOQ potentially could be extended to concentrations lower than 1 ng/mL fexofenadine, this was not necessary for our studies. Uptake of fexofenadine via

Table 3.3: Recovery of Fexofenadine From HEK293 Cell Lysates.

Concentration (ng/mL)	Recovery 1	Recovery 2	Average Recovery
1	75.7	96.3	86.0
2	98.5	116.3	107.4
5	106.8	104.9	105.9
10	119.8	115.5	117.7
50	105.3	77.3	91.3
100	121.4	125.6	123.5
500	117.1	105.7	111.4

transporters like OATP1A2 in transiently transfected cell models, similar to the model we employ, resulted in maximum fexofenadine concentrations of 1-3 μg/mL (Kim 2001); therefore, our method provides a level of sensitivity that will allow the study of even low efficiency transporter systems. This method also meets the FDA guidelines for bioanalysis, with demonstrated accuracy within 90-110% of actual concentrations, precision of ± 10%, and recoveries greater than 75% from the HEK293 cell lysates (FDA 2001).

The choice of the internal standard for this method was a key step in the development and validation. Our first approach was to use the pro-drug terfenadine as internal standard, since it shares extensive structural identity with fexofenadine. We found, however, that the lack of the carboxyl group present in fexofenadine resulted in profoundly different chromatographic behavior, and that we experienced substantial carryover with terfenadine. These problems ruled out the use of terfenadine as internal standard. We then examined another antihistamine, cetirizine, as a possible internal standard based on similar structure and chemical properties with fexofenadine. Use of cetirizine provided very similar chromatographic properties to those of fexofenadine and we observed no carryover or interference with the MRM channels between compounds. These observations supported our use of cetirizine as the internal standard for this assay.

Although examination of fexofenadine transport is one goal of our studies, an additional goal is to employ fexofenadine as a probe for the detection and characterization of drug-drug interactions at specific transporters. This will require the detection and quantitation of potentially competing drugs from cell lysate samples. Our use of gradient elution is intended to support not only the analysis for fexofenadine, but also additional drugs for interaction studies. We have performed initial studies to expand this assay to include the quantitation of four additional probe drugs: buspirone, caffeine, dextromethorphan, and losartan. Use of a steep chromatographic gradient allows us to analyze five drugs and one internal standard in less than five minutes. Once the method is validated for these additional analytes it will allow us to characterize the kinetics of multiple substrates with transporters in a single sample and analytical run, greatly facilitating our investigations of drug-drug interactions.

This gradient method has been successfully used to characterize the kinetics of fexofenadine transport by HEK293 cells transiently-transfected with OATP1A2. This validated method provides a rapid, sensitive, and specific analytical tool supporting the use of fexofenadine as a probe to study the contributions of individual membrane transporters in uptake and efflux of xenobiotics.

3.5: Conclusion

We have presented a fully validated LC-MS/MS method for the quantification of fexofenadine in HEK293 cell lysate from *in vitro* transporter studies according to the

FDA guidelines for bioanalysis (FDA 2001). Using a positive electrospray ionization mode, along with a commercially available internal standard (cetirizine), we have shown this method to be precise, accurate, and both sensitive and selective for the quantification of fexofenadine from cell culture uptake studies. An easy and rapid sample preparation method and quick analysis time (less than 5 minutes) makes this suitable for high throughput bioanalysis.

Chapter 4

Fexofenadine Drug-Drug Interactions at Organic Anion Transporting Polypeptide 1A2

4.1: Abstract

Fexofenadine, a well-tolerated drug with pharmacokinetics controlled by transporters, may be a useful *in vivo* probe for human transporter function. There is, however, uncertainty regarding the roles of specific transporters in fexofenadine pharmacokinetics. We previously tested a probe drug cocktail containing fexofenadine, buspirone, caffeine, dextromethorphan, and losartan for the measurement of transporter and cytochrome P450 activities in humans. A pre-systemic drug-drug interaction was observed, resulting in a decrease in fexofenadine bioavailability when administered as part of the cocktail. We have characterized fexofenadine transport by the two major intestinal OATP uptake transporters *in vitro* and have replicated the fexofenadine-probe drug interaction observed *in vivo*. We conducted *in vitro* studies in OATP1A2-expressing HEK293 cells and OATP2B1-expressing CHO cells. We found that fexofenadine is transported by OATP1A2 (K_m = 35 μM) but that fexofenadine is not effectively transported by OATP2B1. Additional studies demonstrated that losartan, dextromethorphan, and buspirone each inhibited transport of fexofenadine by OATP1A2. The efficient transport of fexofenadine by the intestinal uptake transporter OATP1A2 suggests that this transporter may be a major determinant of the

pharmacokinetics of oral fexofenadine. Moreover, the inhibition of OATP1A2-dependent transport of fexofenadine by three of the drugs in our probe drug cocktail explains the decrease in fexofenadine bioavailability seen in the clinical study. Facile fexofenadine transport and the replication of the fexofenadine-probe drug interaction in this model system supports our conclusion that OATP1A2 is the major uptake transporter for fexofenadine absorption, and suggests that fexofenadine may be an effective probe drug for this transporter.

4.2: Introduction

Drug transporters like the members of the organic anion transporting polypeptide (OATP in humans, Oatp in other species) family play an important role in the absorption, distribution, and excretion of drugs. OATPs are members of the *SLCO* superfamily, mediate the uptake of a wide number of substrates and have overlapping substrate specificity. OATPs can facilitate cellular uptake, and thus enhance intestinal absorption, hepatic uptake, and uptake across the blood brain barrier of drugs and xenobiotics (Hagenbuch and Gui, 2008). Among the eleven members of the OATP superfamily in humans OATP1A2 and 2B1 are the only OATPs associated with drug transport in the enterocyte (Hagenbuch and Meier, 2004; Glaeser et al., 2007).

Fexofenadine pharmacokinetics are controlled by transporters rather than by metabolism (Lippert et al., 1995), suggesting that the pharmacokinetics of orally-

administered fexofenadine could serve as an experimental probe of transporter activity *in vivo*, particularly in the enterocyte. We began to investigate fexofenadine as a probe of transporter function by incorporating it into a cocktail with additional probe drugs for measurement of cytochrome P450 (CYP) activities. These probe drugs (and the corresponding CYPs) were caffeine (CYP1A2), losartan (CYP2C9), dextromethorphan (CYP2D6), and buspirone (CYP3A4). Initial validation of this probe cocktail included testing for pharmacokinetic interactions between the various probe drugs. Transporter function was assessed based on the fexofenadine area under the curve (AUC).

When fexofenadine AUC was compared in 14 subjects after administration of fexofenadine alone with administration of fexofenadine as part of the 5-drug cocktail, half of the subjects showed a 50% or greater decrease in the AUC for fexofenadine in the presence or the cocktail (Chapter 1). Based on this observation we hypothesized that an enterocyte expressed OATP was involved in the absorption of fexofenadine, and that various CYP probes would interact with fexofenadine uptake and thus alter its absorption. We tested this hypothesis by characterizing fexofenadine uptake using OATP1A2 and 2B1 expressing cell lines and determined the inhibitory effects of the probe drugs on this uptake.

4.3: Results

4.3.1: Inhibition of Estrone-3-Sulfate Transport by OATP1A2 in the Presence of Probe Drugs.

In order to determine the effects of the probe drug cocktail components on OATP-mediated transport, we quantified uptake of the model substrate estrone-3-sulfate in the absence and presence of each probe drug into OATP-expressing cells. HEK293 cells transiently expressing human OATP1A2 were exposed to 0.1 μM ^{3}H-estrone-3-sulfate alone or with 10 or 100 μM fexofenadine, losartan, dextromethorphan, buspirone, or caffeine for 30 seconds at pH 7.4 (Figure 4.1). Caffeine had no effect at either concentration, however each of the other four drugs showed concentration dependent inhibition of estrone-3-sulfate uptake. Fexofenadine, losartan and buspirone only inhibited estrone-3-sulfate uptake significantly at 100 μM. Dextromethorphan was the strongest inhibitor resulting in 75% and 85% inhibition at 10 and 100 μM, respectively.

4.3.2: Inhibition of Estrone-3-Sulfate Transport by OATP2B1 at Two pHs in the Presence of Probe Drugs.

The other OATP that is expressed in enterocytes is OATP2B1. Therefore we quantified the effects of the probe drugs on estrone-3-sulfate uptake into cells expressing OATP2B1. Experiments were performed at pH 7.4 and pH 5.5 since the range of substrates for OATP2B1 has been reported to be significantly broader at the lower pH (Kobayashi et al., 2003). In contrast to OATP1A2, OATP2B1-mediated uptake of estrone-3-sulfate was only inhibited in the presence of 10 and 100 μM losartan while none of the other probe drug cocktail components showed any effect at either pH 7.4 (Figure 4.2.1) or pH 5.5 (Figure 4.2.2).

4.3.3: Fexofenadine Transport by OATP1A2 and OATP2B1.

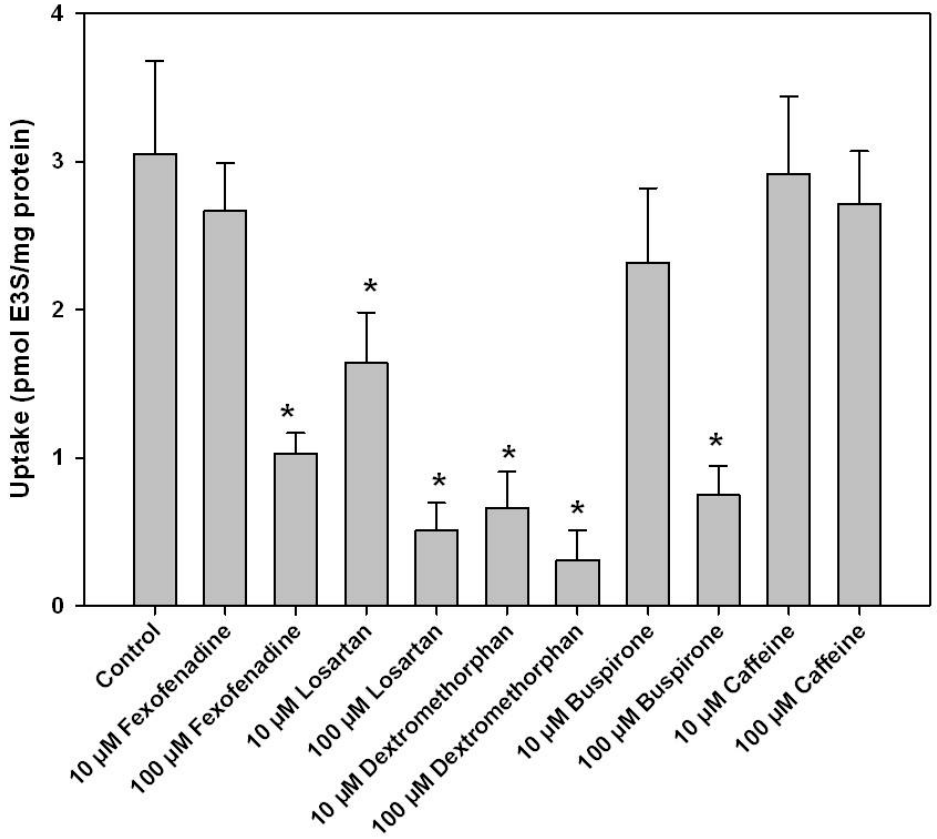
Given that drugs in the probe cocktail were capable of inhibiting OATP-mediated estrone-3-sulfate uptake and given that both OATP1A2 and OATP2B1 have been reported to transport fexofenadine, we next quantified fexofenadine uptake into OATP1A2- and OATP2B1-expressing cells. HEK293 cells transiently expressing human OATP1A2 (Figure 4.3.1) or CHO cells stably expressing OATP2B1 (Figure 4.3.2) were exposed to 1, 10, and 100 μM fexofenadine at pH 7.4 for 10 minutes and uptake was measured. Uptake mediated by HEK293 cells that expressed OATP1A2

was clearly higher than uptake by the control HEK293 cells transfected with empty vector at all three concentrations measured (Figure 4.3.1). Net uptake increased with increasing concentrations from about 10 to 150 pmoles and seemed to reach saturation. OATP2B1 mediated uptake however was between 100 and 150 fold lower (between 0.2 and 3 pmoles) (Figure 4.3.2). Although this uptake seemed to increase with increasing concentrations of fexofenadine, the absolute level was too low to determine the kinetic parameters and suggested that OATP2B1 may not be a major contributor to fexofenadine uptake system in enterocytes.

4.3.4: Fexofenadine Transport Kinetics by OATP1A2.

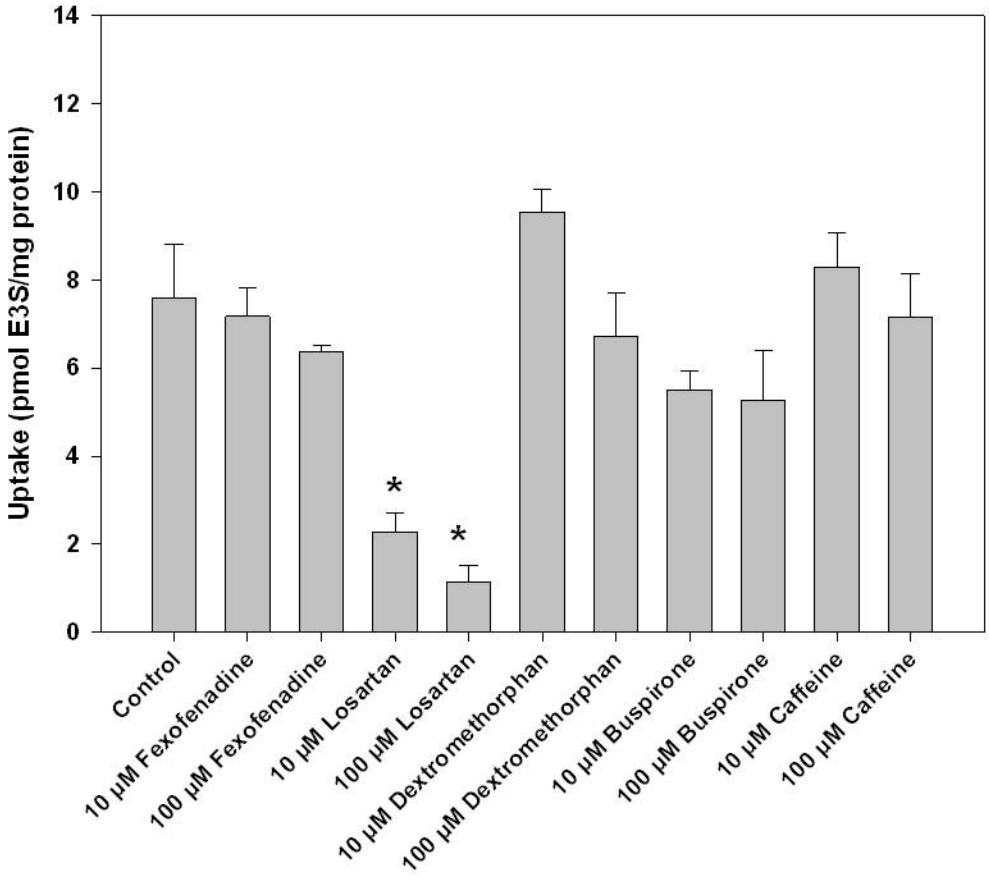
In order to characterize the transport kinetics of fexofenadine uptake by OATP1A2 we first determined the initial linear time range. Uptake was linear up to 2.5 minutes and therefore subsequent kinetic experiments were done at the 2 minute time point. OATP1A2-expressing and control cells expressing only the empty vector were incubated with increasing concentrations of fexofenadine from 0.5-50 μM. After subtracting the values obtained with the control cells, net OATP1A2-mediated transport showed clear saturation kinetics with a K_m value of 35.1 μM and a V_{max} value of 21 pmol/mg*min for fexofenadine uptake (Figure 4.4).

Figure 4.1:



Multiple Probe Drugs Inhibit OATP1A2-Mediated Transport of Estrone-3-Sulfate. HEK293 cells transiently expressing human OATP1A2 were exposed to 0.1 µM ^{3}H-estrone-3-sulfate (0.4 µCi/mL) alone or with 10 or 100 µM fexofenadine, losartan, dextromethorphan, buspirone, or caffeine for 30 seconds at pH 7.4. After correction for protein, uptake into empty vector was subtracted to determine the net OATP1A2-mediated uptake. Values are expressed as pmol E3S/mg protein; each value is the mean ± SD of two independent experiments. Asterisks represent statistically significant differences from the DMSO control (*, $p < 0.05$).

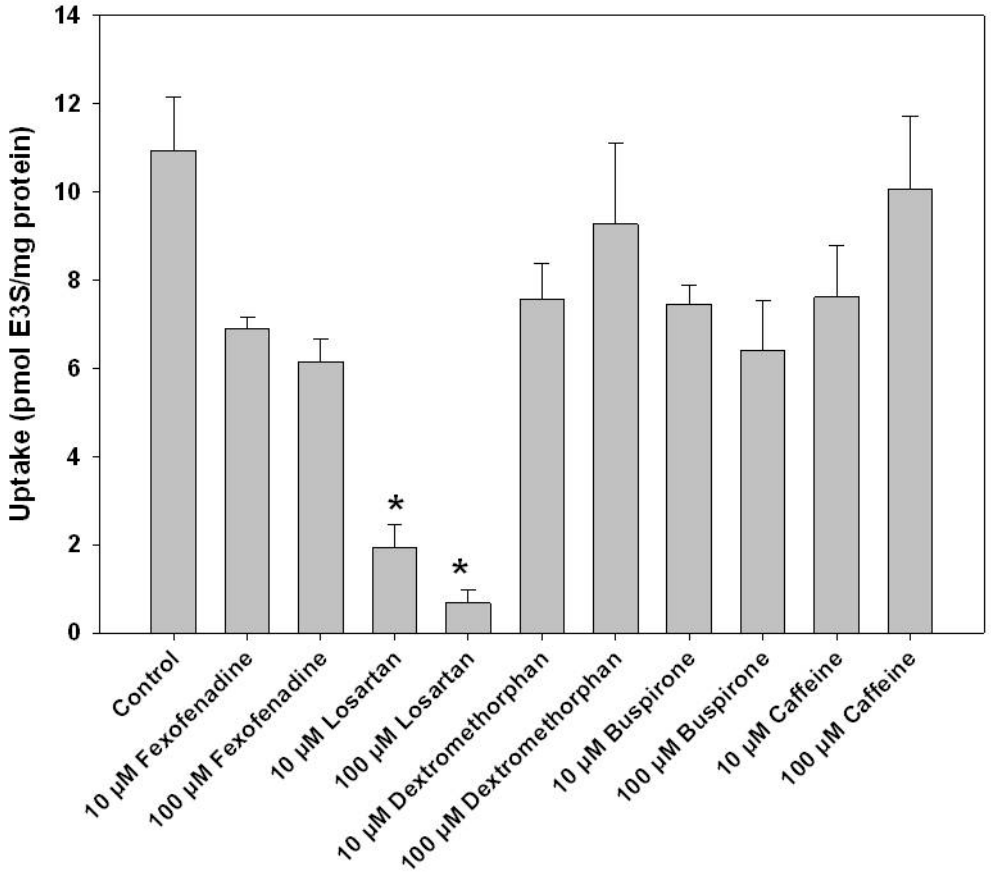
Figure 4.2.1:



Losartan Inhibits OATP2B1-Mediated Transport of Estrone-3-Sulfate at pH 7.4.

CHO cells expressing human OATP2B1 were exposed to 0.1 µM ^{3}H-E3S (0.4 µCi/mL) alone or with 10 or 100 µM fexofenadine, losartan, dextromethorphan, buspirone, or caffeine for 20 seconds at pH 7.4. After correction for protein, uptake into wild-type control cells was subtracted to determine the net OATP2B1-mediated uptake at pH 7.4. Values are expressed as pmol E3S/mg protein; each value is the mean ± SD of two independent experiments. Asterisks represent statistically significant differences from the DMSO control (*, $p < 0.05$).

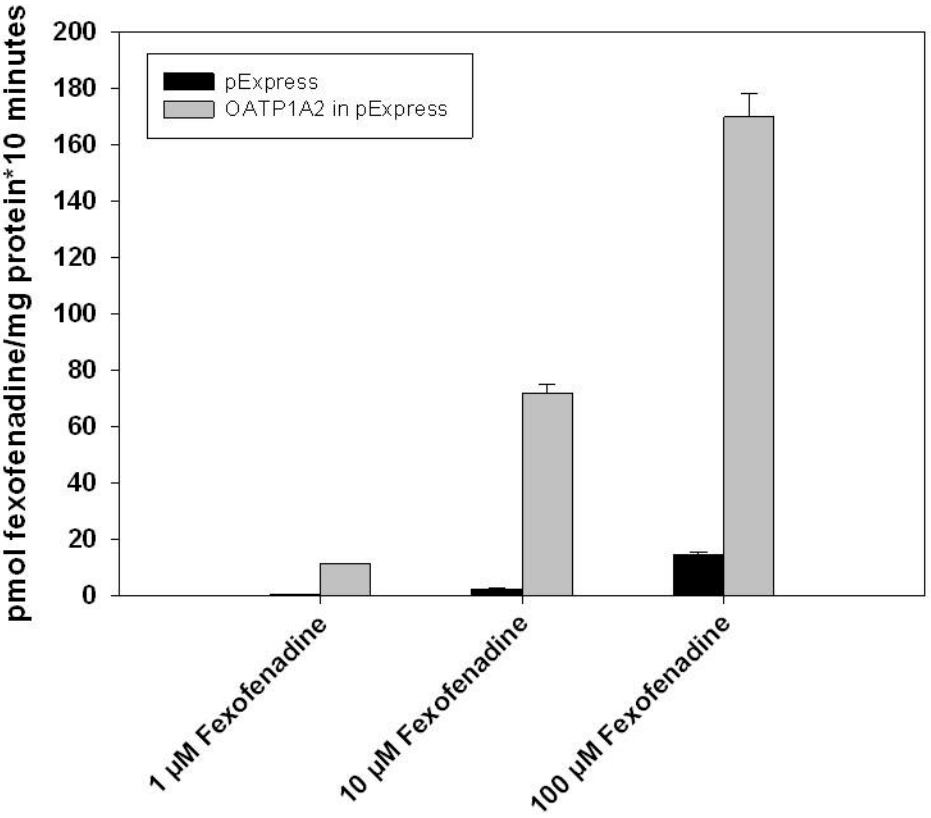
Figure 4.2.2:



Losartan Inhibits OATP2B1-Mediated Transport of Estrone-3-Sulfate at pH 5.5.

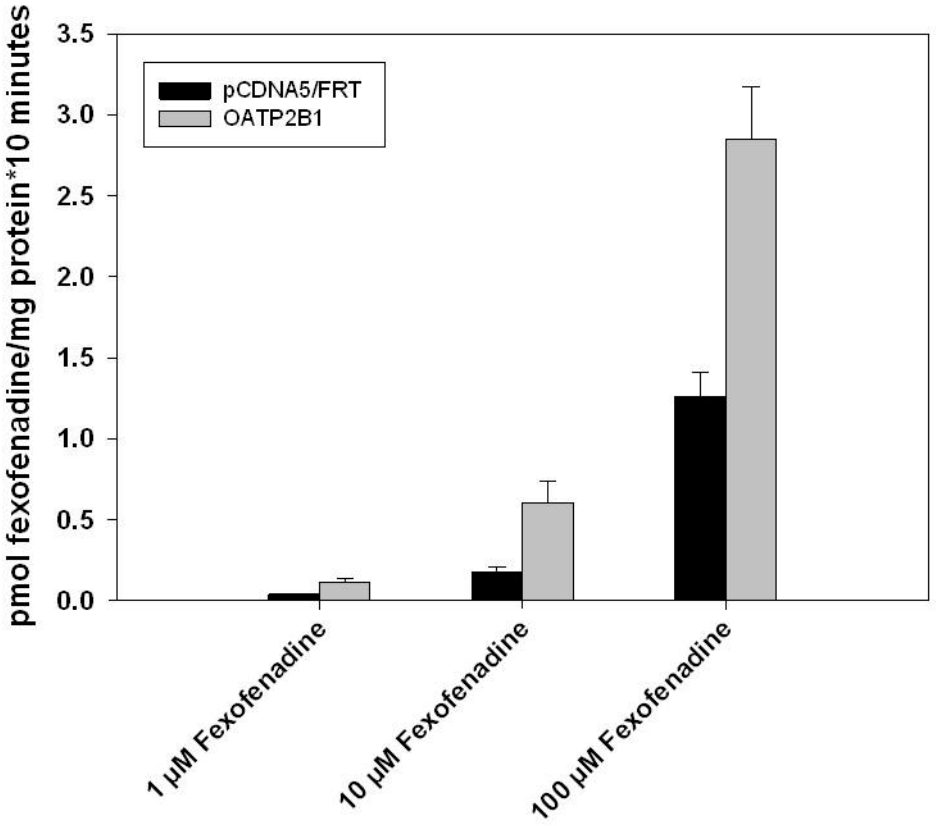
CHO cells expressing human OATP2B1 were exposed to 0.1 µM ^{3}H-E3S (0.4 µCi/mL) alone or with 10 or 100 µM fexofenadine, losartan, dextromethorphan, buspirone, or caffeine for 20 seconds at pH 5.5. After correction for protein, uptake into wild-type control cells was subtracted to determine the net OATP2B1-mediated uptake at pH 5.5. Values are expressed as pmol E3S/mg protein; each value is the mean ± SD of two independent experiments. Asterisks represent statistically significant differences from the DMSO control (*, $p < 0.05$).

Figure 4.3.1:



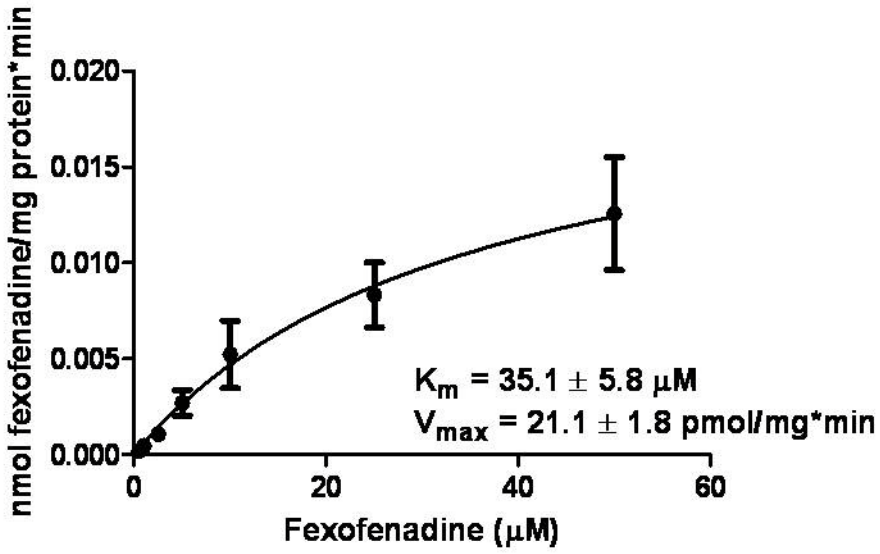
Fexofenadine Transport by OATP1A2. HEK293 cells transiently expressing human OATP1A2 were exposed to 1, 10, and 100 µM fexofenadine at pH 7.4 for 10 minutes to determine uptake of fexofenadine. Uptake was corrected for empty vector and normalized to protein. Fexofenadine appears to be transported by OATP1A2 in a concentration-dependent manner (Graph is a representative from at least three independent experiments; each point is an average of 3 wells ± SD).

Figure 4.3.2:



Fexofenadine Transport by OATP2B1. CHO cells expressing human OATP2B1 were exposed to 1, 10, and 100 µM fexofenadine at pH 7.4 for 10 minutes to determine uptake of fexofenadine. Uptake was corrected for empty vector and normalized to protein. Fexofenadine appears to be minimally transported by OATP2B1 and not statistically significant (Graph is a representative from at least three independent experiments; each point is an average of 3 wells ± SD).

Figure 4.4:

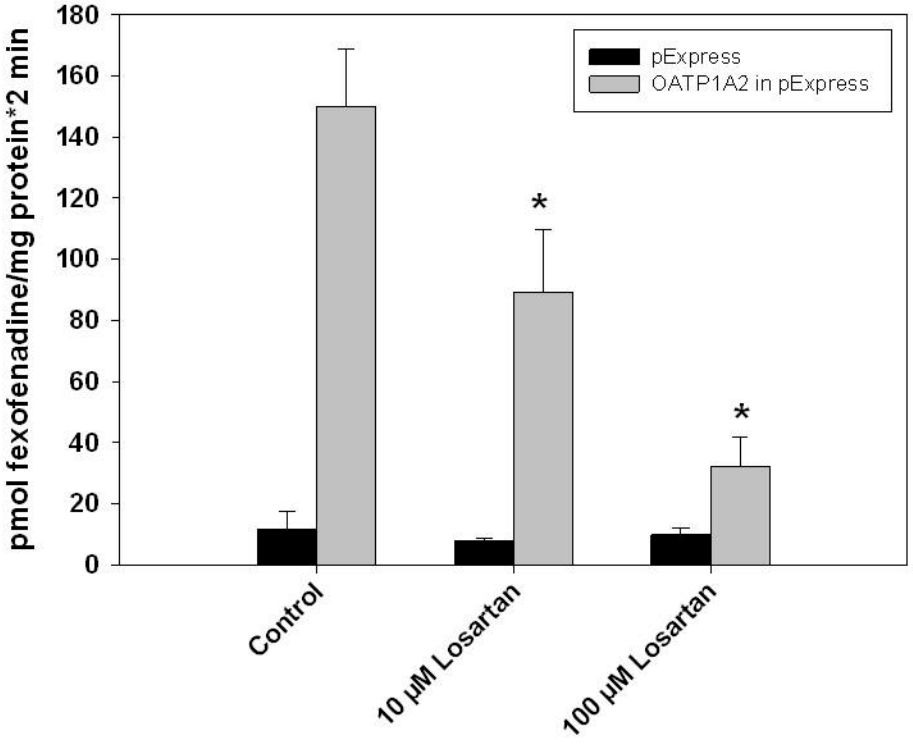


Concentration-Dependent Fexofenadine Uptake by OATP1A2. HEK293 cells transiently expressing human OATP1A2 were exposed for 2 minutes to increasing concentrations of fexofenadine from 0.5-50μM. Time dependent uptake of fexofenadine was linear over 2.5 minutes (data not shown). Michaelis-Menten curve fitting was used to determine the K_m value of 35.1± 5.8 μM with a V_{max} of 21 ± 0.0018 pmol/mg*min (Graph representative of one assay, average of 3 wells ± SD; K_m value representative of 5 assays ± SEM).

4.3.5: Probe Drug Cocktail Components Inhibit Fexofenadine Transport by OATP1A2.

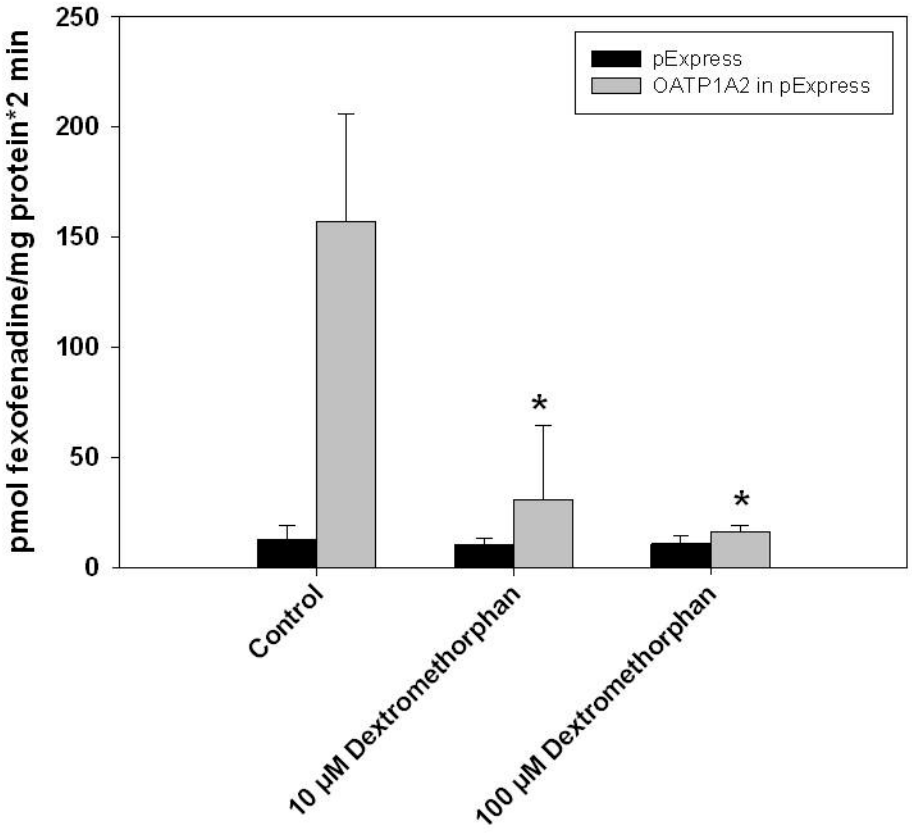
Given that OATP1A2 seems to play a dominant role in the uptake of fexofenadine, we examined the effects of the other probe drugs at 10 and 100 μM on OATP1A2-mediated fexofenadine uptake. OATP1A2-expressing cells were exposed to 30 μM fexofenadine alone (control) and in the presence of 10 or 100 μM losartan (Figure 4.5.1), dextromethorphan (Figure 4.5.2), or buspirone (Figure 4.5.3). Both 10 and 100 μM losartan significantly inhibited OATP1A2-mediated uptake of fexofenadine in a concentration dependent manner. Ten and 100 μM dextromethorphan also significantly inhibited OATP1A2-mediated uptake of fexofenadine. Only 100 μM buspirone inhibited OATP1A2-mediated uptake of fexofenadine in a significant manner as compared to the effects of losartan and dextromethorphan.

Figure 4.5.1:



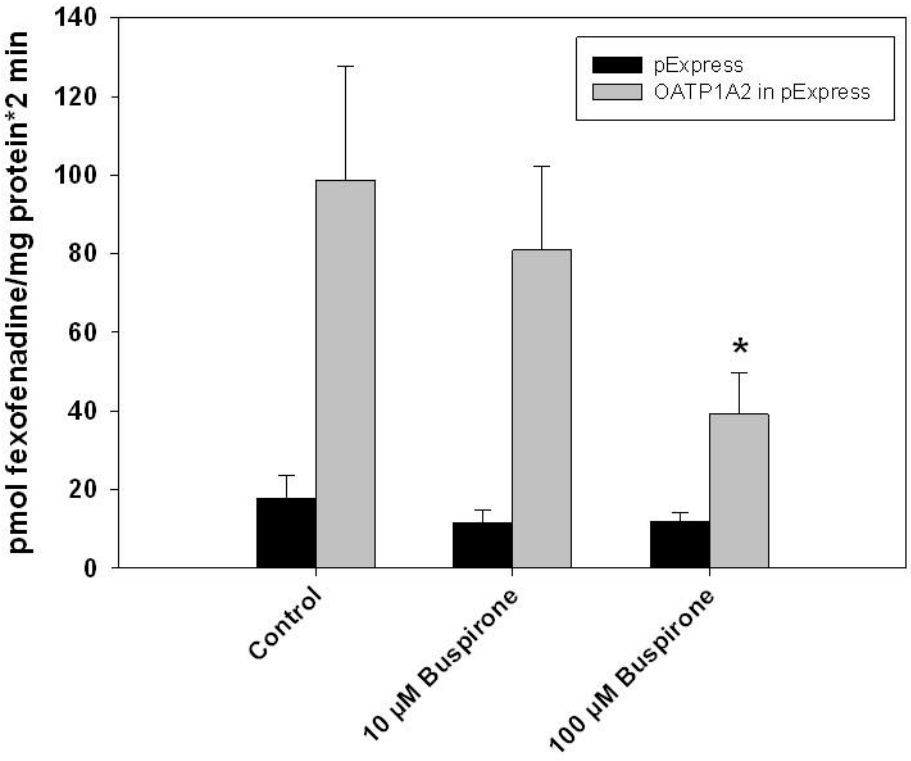
Concentration-Dependent Inhibition of the OATP1A2-Mediated Uptake of Fexofenadine by Losartan. Uptake of 30 μM fexofenadine was measured alone and in the presence of 10 or 100 μM losartan for two minutes. Uptake of fexofenadine by cells expressing empty vector was subtracted from uptake by cells expressing OATP1A2 and normalized to protein. Asterisks represent statistically significant differences from the 30 μM fexofenadine control (*, $p < 0.05$).

Figure 4.5.2:



Concentration-Dependent Inhibition of the OATP1A2-Mediated Uptake of Fexofenadine by Dextromethorphan. Uptake of 30 µM fexofenadine was measured alone and in the presence of 10 or 100 µM dextromethorphan for two minutes. Uptake of fexofenadine by cells expressing empty vector was subtracted from uptake by cells expressing OATP1A2 and normalized to protein. Asterisks represent statistically significant differences from the 30 µM fexofenadine control (*, $p < 0.05$).

Figure 4.5.3:



Concentration-Dependent Inhibition of the OATP1A2-Mediated Uptake of Fexofenadine by Buspirone. Uptake of 30 μM fexofenadine was measured alone and in the presence of 10 or 100 μM buspirone for two minutes. Uptake of fexofenadine by cells expressing empty vector was subtracted from uptake by cells expressing OATP1A2 and normalized to protein. Asterisks represent statistically significant differences from the 30 μM fexofenadine control (*, $p < 0.05$).

4.4: Discussion:

The major findings of the present study are the demonstration that: 1) OATP1A2 is by far the most effective of the intestinal OATPs for facilitation of fexofenadine absorption, and 2) that the probe drug cocktail components losartan, dextromethorphan, and buspirone each can inhibit fexofenadine transport mediated by OATP1A2 in a concentration dependent manner.

The first finding supports the hypothesis that OATP1A2 is the dominant transporter of fexofenadine in the enterocyte and is in agreement with the report of Cvetkovic et al. (1999) and Glaeser et al. (2007). We were unable to characterize transport of fexofenadine by the other enterocyte-expressed OATP, namely OATP2B1 (Figure 4.3.2). Net transport of fexofenadine by OATP2B1-expressing cells was 50 to 150 times less than fexofenadine transport seen in OATP1A2-expressing cells. This is in marked contrast to the transport of the model substrate estrone-3-sulfate in these same OATP-expressing cells, where OATP2B1 was the more effective transporter (Figure 4.2.1 and 4.2.2). Finally, we did not observe either a linear time dependence or clear concentration-dependent uptake of fexofenadine by OATP2B1, observations that would be expected if a saturable transporter was present.

Our findings with cells expressing OATP2B1 are in agreement with the report of Shimizu et al. (2005) who did not see significant transport of fexofenadine by OATP2B1. In addition, Glaeser et al. (2007) saw no uptake of fexofenadine by OATP2B1 in comparison to vehicle control. In contrast, Imanaga et al. (2011) saw significant transport of fexofenadine in injected oocytes at pH 6.5. They proposed a pH-dependence to explain the differences between their result and that of the other laboratories. It should be noted, however, that we examined OATP2B1-dependent fexofenadine uptake at both pH 7.4 and pH 5.5, and saw 50 to 100 times less fexofenadine transported than by its counterpart OATP1A2, regardless of pH. These observations do not support a role for OATP2B1 as a major contributor to fexofenadine uptake from the intestine.

Our characterization of OATP-dependent transport of fexofenadine instead suggests that OATP1A2 is the dominant transporter mediating absorption of this drug from the intestine. The critical role of this transporter in controlling fexofenadine pharmacokinetics overall is supported by comparing the results of our probe drug interaction studies *in vitro* with previous drug-drug interactions *in vivo*. We previously saw a marked decrease in fexofenadine C_{max} and AUC in healthy subjects after administration of a probe drug cocktail containing buspirone, caffeine, dextromethorphan, fexofenadine, and losartan, relative to the values in the same subjects when fexofenadine was administered as a single drug (Reed et al., manuscript in preparation). Neither plasma half-life nor urinary clearance of fexofenadine was altered by the observed drug-drug interaction. This observation indicates a strong pre-

systemic interaction between fexofenadine and one or more of the other probe drugs in the cocktail *in vivo*, resulting in decreased absorption of fexofenadine. The ability to replicate a decrease in fexofenadine transport by OATP1A2 *in vitro* that mimics observations made *in vivo* is consistent with OATP1A2 being the dominant transporter modulating fexofenadine uptake.

Multiple drugs are often dosed orally in humans, increasing the risk of significant drug-drug interactions. As a result of this possible concurrent dosing, it is important to assess potential drug-drug interactions at both drug metabolizing enzymes and at membrane transporters. There are numerous well-characterized probes for several of the major CYPs involved in drug metabolism; however there is a lack of well-tolerated and well-characterized probes for measurement of transporter function and drug-drug interactions *in vivo* (Frye et al., 1997; Christensen et al., 2003; Ryu et al., 2007). Fexofenadine is a potential probe for transporter activity based on the fact that it is not metabolized and is excreted unchanged. Based on our findings reported here, it is reasonable to interpret decreases in both C_{max} and AUC of oral fexofenadine, in the absence of changes in clearance, as a result of OATP1A2 inhibition. Conversely, stimulation of P-gp would give the same result and such allosteric stimulation has been reported (Parasrampuria et al., 2001), however, P-gp inhibition is more widely documented as a drug-drug interaction (Wang et al., 2002; Dresser et al. 2003, Kamath et al. 2005, Yasui-Furukori et al. 2005, Shimizu et al., 2006) resulting in an increased C_{max} and AUC for fexofenadine. Acute drug-drug interactions resulting in decreased AUC for fexofenadine thus are most readily explained as resulting from OATP1A2

inhibition, whereas interactions increasing fexofenadine AUC would result from P-gp inhibition. However if both OATP1A2 activity and P-gp activity are affected simultaneously by a drug-drug interaction, then the interpretation of our fexofenadine results are not as clear. In addition, our interpretation is limited to acute, direct drug-drug interactions. Interpretation of results from chronic exposures, where not only direct interactions but effects on transporter expression also may be involved, would be even more complicated.

Qiang et al. (2009) reported a fexofenadine-drug interaction in rats similar to what we have observed. Oral dosing rats with the combination of fexofenadine and fluvastatin decreased fexofenadine AUC by 17-50% in rats, which is quite similar to our clinical findings. The concurrent use of fluvastatin did not affect the intravenous pharmacokinetics of fexofenadine. The marked decrease in oral AUC and C_{max} values of fexofenadine with no change in t_{max} or $t_{1/2}$ with fluvastatin pretreatment is likely due to the reduced intestinal absorption of fexofenadine rather than the enhanced systemic elimination by efflux transporters. They hypothesize that the interaction seen between fexofenadine and fluvastatin may be occurring at an OATP since both drugs are substrates of multiple OATPs. Our current study involving fexofenadine and the probe drug cocktail is the first to show an *in vitro* fexofenadine-drug interaction at an OATP that could explain an *in vivo* drug interaction in human subjects (Figure 4.5.1, 4.5.2, and 4.5.3).

Fexofenadine interactions with dietary components also have been reported. Dresser et al. (2002 and 2005) have shown that fruit juices decrease oral bioavailability of fexofenadine. It is likely that oral co-administration of fruit juice and fexofenadine results in a decreased AUC due to inhibition of OATP transporters in the small intestine. Concominant administration of fruit juice with oral fexofenadine creates a transient inhibitory effect; however it does not decrease expression of OATP1A2 at the mRNA level (Dresser et al., 2007). Taken with the fexofenadine-fluvastatin interaction seen in rats as well as our own clinical observations, the fexofenadine-probe cocktail interaction is most likely to be occurring at an OATP in the enterocyte.

Our results suggest that fexofenadine is transported predominantly by OATP1A2 in the enterocyte. Inhibition of fexofenadine transport by OATP1A2 with multiple components of a probe drug cocktail (losartan, dextromethorphan, buspirone) supports previous clinical observations and should be considered an important, previously unknown set of drug-drug interactions at an uptake transporter. Moreover, these data suggest that we are able to replicate a decrease in fexofenadine transport *in vitro* that mimics observations made *in vivo,* thereby allowing us to determine the contribution of various OATPs to fexofenadine absorption and how drug-drug interactions can alter this absorption. Although there are limitations, oral fexofenadine appears to be a reasonable probe for acute, inhibitory drug-drug- and drug-diet interactions at intestinal OATP1A2.

Chapter 5

Multiple Organic Anion Transporting Polypeptides (OATPs) Play A Role in the Disposition of Losartan

5.1: Abstract

Although losartan clearance is controlled primarily by CYP2C9 activity, transporters are likely to play a role in losartan disposition because several OATPs have been shown to transport angiotensin II receptor antagonists. We first tested losartan modulation of radiolabeled substrate transport with 4 members of the organic anion transporting polypeptides family. Uptake of the model substrates estradiol-17β - glucuronide (E17-βG) and estrone-3-sulfate (E3S) was measured using cell lines expressing each of these OATPs in the absence and presence of losartan. Uptake of E3S by both OATP1A2 and OATP2B1 was inhibited by both 10 μM and 100 μM losartan; however uptake of E3S by OATP1B1 and OATP1B3 was inhibited only by 100 μM losartan. OATP1B1- and OATP1B3-mediated uptake of E17-βG was inhibited by losartan at both 10 and 100 μM losartan. The ability of losartan to inhibit model substrate transport by four major OATPs indicates that losartan could be a substrate of these transporters. Uptake of losartan by OATP1A2, OATP1B1, OATP1B3, and OATP2B1 was saturable at pH 7.4 with K_m values of 58.2, 3.0, 17.6, and 16.6 μM respectively. Apparent OATP2B1-mediated uptake of losartan was higher at pH 5.5 as compared to pH 7.4, but was not saturable. These results demonstrate that losartan is

a substrate of OATP1A2, OATP1B1, 1B3, and 2B1, and thus these transporters may play a significant role in losartan disposition in both the small intestine and liver.

5.2: Introduction

The majority of drugs are dosed orally and in order for them to reach their target sites, they have to be absorbed in the small intestine and travel to the portal vein. These drugs are then transported to the liver and taken up into the hepatocytes where they can be metabolized by many enzymes including those in the cytochrome P450 family (CYP). Although some drugs can cross the plasma membrane by simple diffusion, the uptake of most drugs is controlled by transporters such as the organic anion transporting polypeptides (OATPs) that are expressed in epithelial cells throughout the body. Enterocytes express OATP1A2 and OATP2B1 (Kobayashi et al., 2003 and Glaeser et al., 2007) while in hepatocytes OATP1B1, OATP1B3 and OATP2B1 are expressed (Konig et al 2000a, Konig et al. 2000b, Kullak-Ublick et al. 2001).

Losartan, an angiotensin II receptor (Type AT_1) antagonist is primarily converted by hepatic CYP2C9 to its active metabolite E3174 (Stearns et al., 1995; Yun et al., 1995). Therefore it has been used in various probe drug cocktails as a probe for CYP2C9 activity (Christensen et al., 2003; Ryu et al., 2007). Although conversion to its

active metabolite E3174 provides an index of CYP2C9 activity, transporters may also play a role in losartan disposition. It has been demonstrated that other sartans such as valsartan, telmisartan and olmesartan are substrates of liver-specific OATP1B1 and 1B3 (Yamashiro et al., 2006, Ishiguro et al., 2006, Yamada et al., 2007). Because this family of sartans is structurally similar, it suggested that losartan could also be a substrate of OATPs. In addition, in a recent study where we characterized fexofenadine transport by OATPs (Chapter 4) we could demonstrate that losartan was an inhibitor of OATP-mediated substrate uptake.

Based on these observations, we hypothesized that both enterocyte- and hepatocyte-expressed OATPs could be involved in the uptake and disposition of losartan. We tested this hypothesis by characterizing losartan uptake *in vitro* using cell lines expressing OATP1A2, OATP2B1, OATP1B1, or OATP1B3.

5.3: Results

5.3.1: Losartan Inhibits OATP-Mediated Substrate Transport.

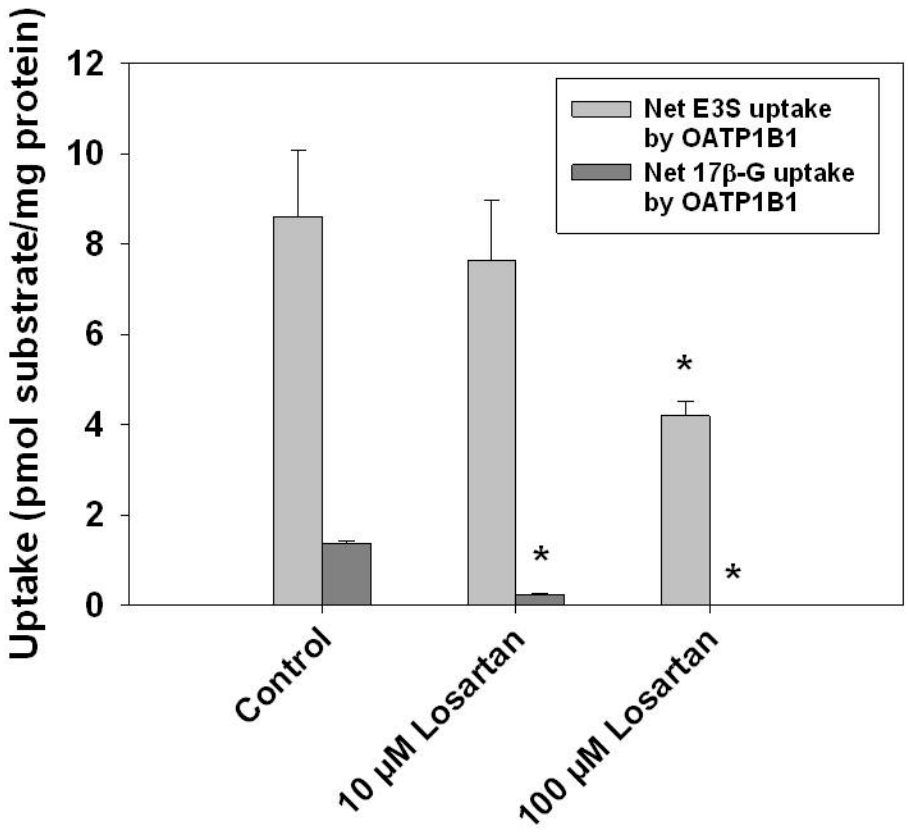
Previously, our lab investigated the effects of a probe drug cocktail containing fexofenadine, losartan, dextromethorphan, buspirone, and caffeine on OATP1A2- and OATP2B1-mediated estrone-3-sulfate transport (Chapter 4). Interestingly, losartan

inhibited both OATP1A2- and OATP2B1-mediated transport of estrone-3-sulfate. Because losartan is metabolized to E3174 in the liver (Stearns et al., 1995; Yun et al., 1995), OATPs expressed in hepatocytes may play a role in hepatocelullar losartan uptake. In order to determine whether the observed inhibition of estrone-3-sulfate was specific for OATP1A2 and OATP2B1, we also quantified uptake of estrone-3-sulfate and estradiol-17β-glucuronide in the absence and presence of losartan into OATP1B1- and 1B3-expressing CHO cells. Uptake into CHO cells stably expressing human OATP1B1 (Figure 5.1.1) or 1B3 (Figure 5.1.2) was inhibited by losartan. While uptake of estradiol-17β glucuronide was inhibited by both 10 and 100μM of losartan, estrone-3-sulfate uptake was only inhibited by 100μM losartan in both OATP1B1 and 1B3 expressing CHO cells.

5.3.2: Losartan is Transported by Multiple OATPs.

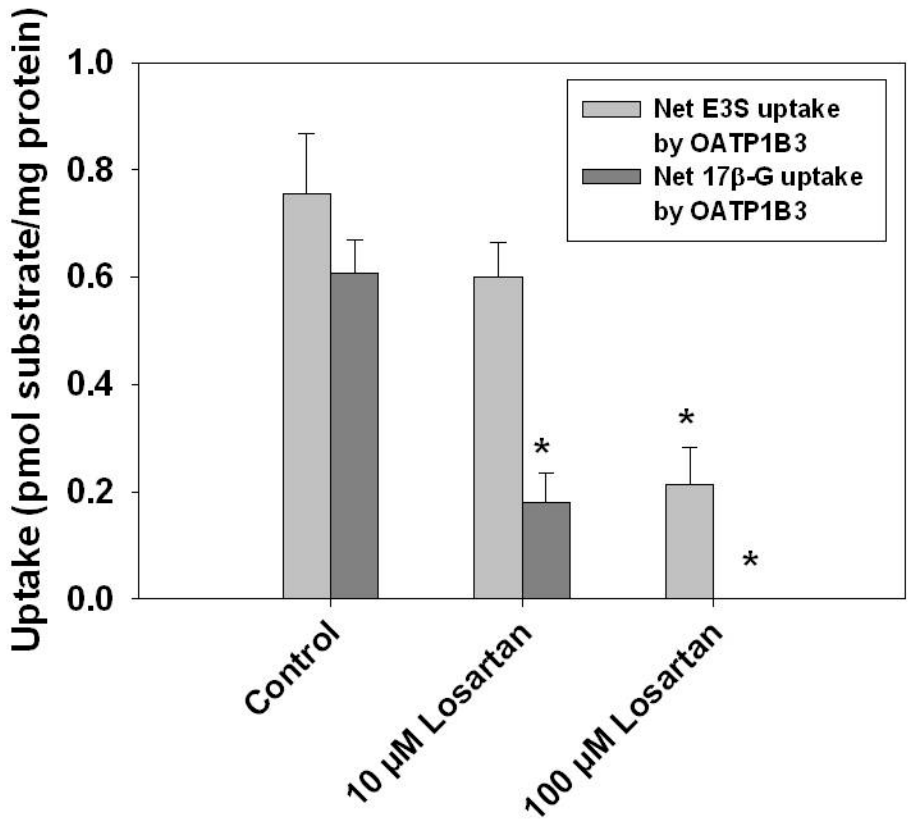
Given that losartan was capable of inhibiting substrate uptake by all four OATPs, we next tested whether losartan would be taken up by OATP1A2-, OATP2B1-, OATP1B1-, and OATP1B3-expressing cells. Figure 5.2.1 illustrates that losartan is taken up into HEK293 cells transiently expressing human OATP1A2 in a concentration-dependent manner. In Figure 5.2.2 demonstrates that CHO cells stably expressing OATP2B1 (A, pH 7.4 and B, pH 5.5) also transport losartan in a concentration-dependent manner, with greater losartan transport at pH 5.5. OATP1B1 (A) and OATP1B3 (B) also transport losartan in a similar manner (Figure 5.2.3).

Figure 5.1.1:



Losartan Inhibits OATP1B1-Mediated Transport of Estrone-3-Sulfate and Estradiol-17β-Glucuronide. CHO cells stably expressing human OATP1B1 were exposed to 0.1 μM ^{3}H-estrone-3-sulfate (0.4 μCi/mL) or 0.1 μM ^{3}H-estradiol-17β-glucuronide (0.4 μCi/mL) alone or with 10 or 100 μM losartan for 20 seconds. After correction for protein, uptake into wild-type CHO cells was subtracted to determine the net estrone-3-sulfate (light gray bars) or estradiol-17β-glurcuronide (dark gray bars). Values are the mean ± SD of two independent experiments.

Figure 5.1.2:



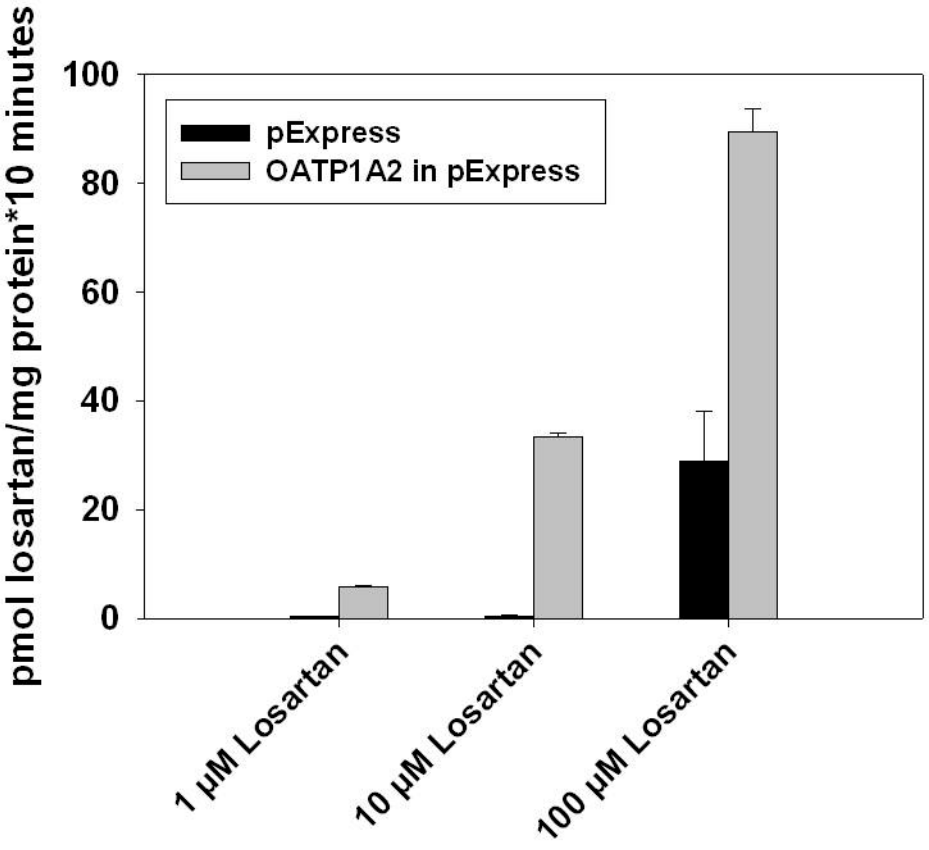
Losartan Inhibits OATP1B3-Mediated Transport of Estrone-3-Sulfate and Estradiol-17β-Glucuronide. CHO cells stably expressing human OATP1B3 were exposed to 0.1 μM ^{3}H-estrone-3-sulfate (0.4 μCi/mL) or 0.1 μM ^{3}H-estradiol-17β-glucuronide (0.4 μCi/mL) alone or with 10 or 100 μM losartan for 20 seconds. After correction for protein, uptake into wild-type CHO cells was subtracted to determine the net estrone-3-sulfate (light gray bars) or estradiol-17β-glurcuronide (dark gray bars). Values are the mean ± SD of two independent experiments.

Net uptake increased with increasing concentrations of losartan for all OATPs tested and appeared to reach saturation. These results demonstrate that losartan is a substrate of the four tested OATPs.

5.3.3: Losartan Kinetics by OATPs Expressed in the Enterocyte.

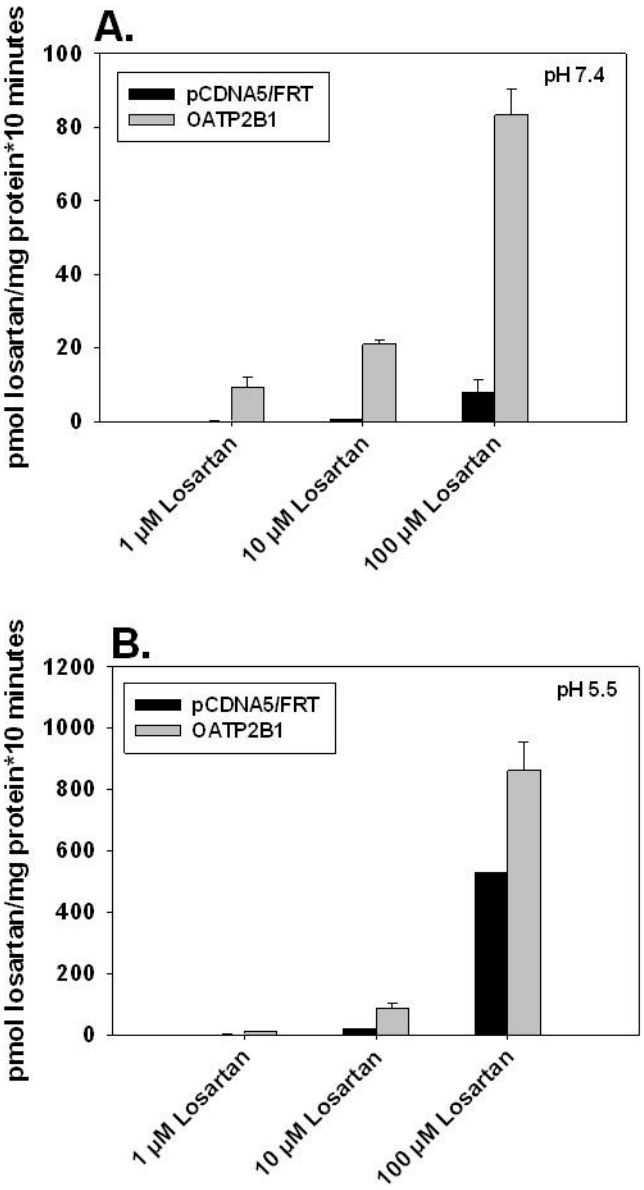
Next we characterized the transport kinetics of losartan by OATP1A2 and OATP2B1 which are expressed in enterocytes. We first established the initial linear range of uptake which was 40 seconds for OATP1A2 and 1 minute for OATP2B1. Therefore, concentration dependent uptake of losartan was determined at 20 seconds for OATP1A2 and 30 seconds for OATP2B1. In Figure 5.3 A, kinetics of OATP1A2-mediated losartan uptake are shown. Transport showed clear saturation with a K_m value of 58.2 μM and a V_{max} value of 12 pmol/mg*min. For OATP2B1, a K_m value of 16.6 μM and a V_{max} value of 14 pmol/mg*min was determined (Figure 5.3, B).

Figure 5.2.1:



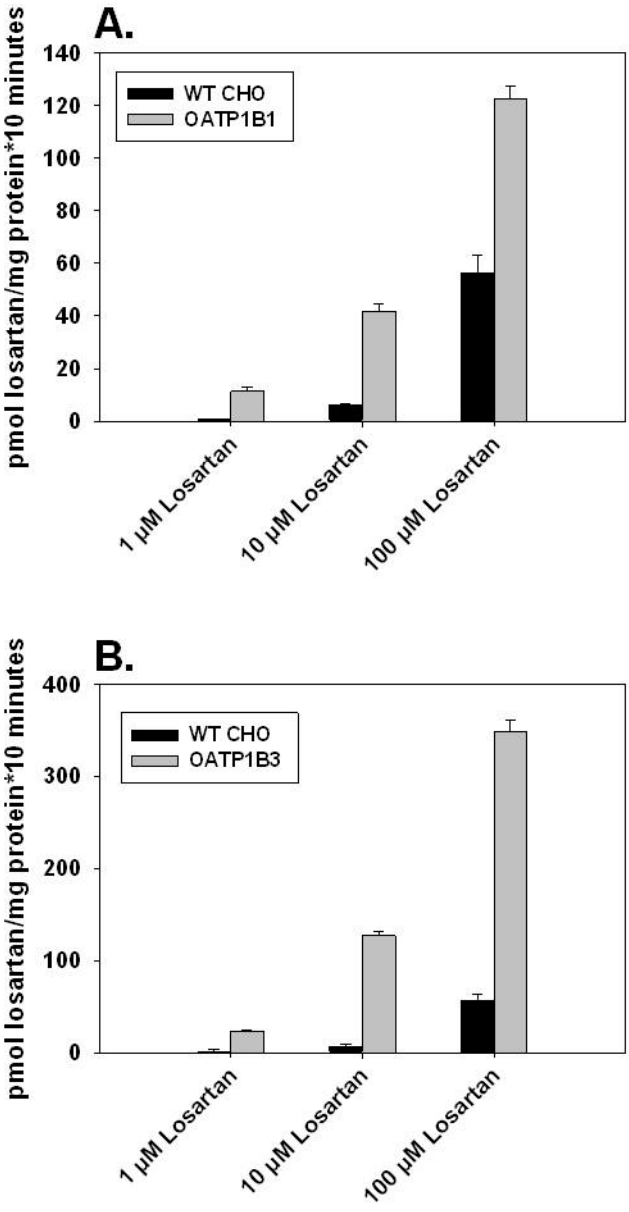
Losartan is a Substrate of OATP1A2. HEK293 cells transiently expressing human OATP1A2 were exposed to 1, 10, and 100 µM losartan for 10 minutes and uptake was determined. Uptake was normalized to protein. Values are the mean ± SD of three independent experiments.

Figure 5.2.2:



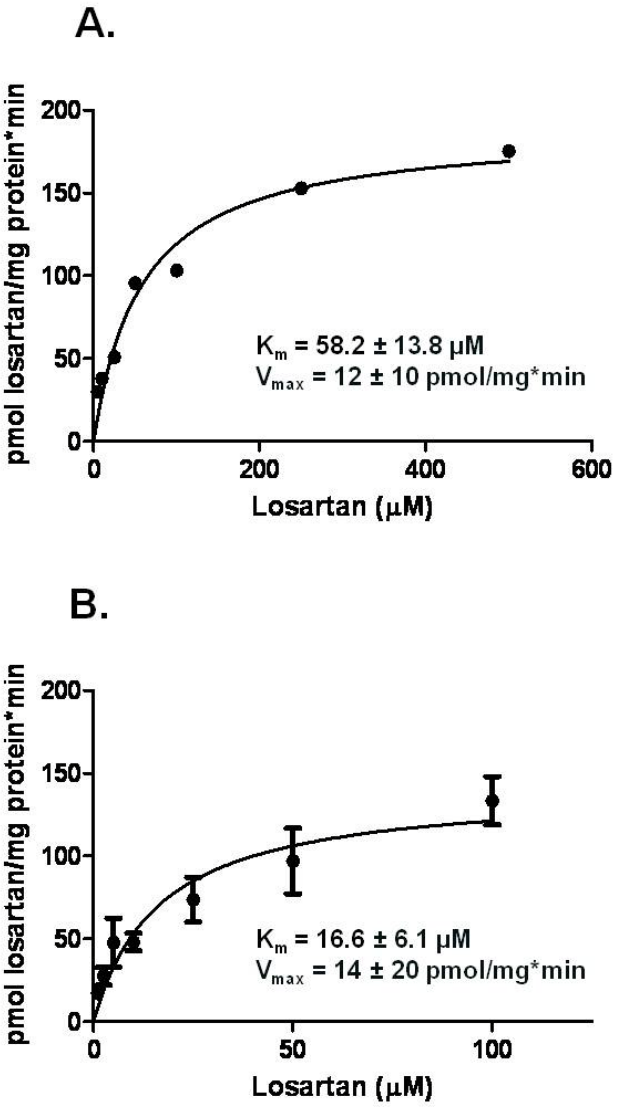
Losartan is a Substrate of OATP2B1 at Two pHs. CHO cells expressing human OATP2B1 were exposed to 1, 10, and 100 µM losartan for 10 minutes at pH 7.4 (A) or pH 5.5 (B) and uptake was determined. Uptake was normalized to protein. Values are the mean ± SD of three independent experiments.

Figure 5.2.3:



Losartan is a Substrate of OATP1B1 and 1B3. CHO cells expressing human OATP1B1 (A) or OATP1B3 (B) were exposed to 1, 10, and 100 μM losartan for 10 minutes and uptake was determined. Uptake was normalized to protein. Values are the mean ± SD of three independent experiments.

Figure 5.3:



Concentration-Dependent Uptake of Losartan by OATP1A2 and OATP2B1. Cells expressing human OATP1A2 (A) were exposed for 30 seconds to 5-500 μM losartan. Cells expressing human OATP2B1 (B) were exposed for 30 seconds to 1-100 μM losartan at pH 7.4. Michaelis-Menten curve fitting was used to determine the K_m values of 58.2 μM for OATP1A2 and 16.6 μM for OATP2B1. Uptake was corrected for empty vector and normalized to protein. Graphs representative of one assay, average of 3 wells ± SD; K_m value representative of 3 assays ± SEM.

5.3.4: Losartan Kinetics by OATPs Expressed in the Hepatocyte.

For OATP1B1- and OATP1B3-mediated uptake of losartan, transport was linear over the first minute and therefore experiments were performed at 30 seconds. Figure 5.4 shows that after subtracting the values obtained with the control cells, net OATP1B1-mediated transport showed clear saturation kinetics with a K_m value of 3.0 μM and a V_{max} value of 20 pmol/mg*min (A), whereas OATP1B3-mediated uptake of losartan had a K_m value of 17.6 μM and a V_{max} value of 120 pmol/mg*min (B).

Figure 5.4:

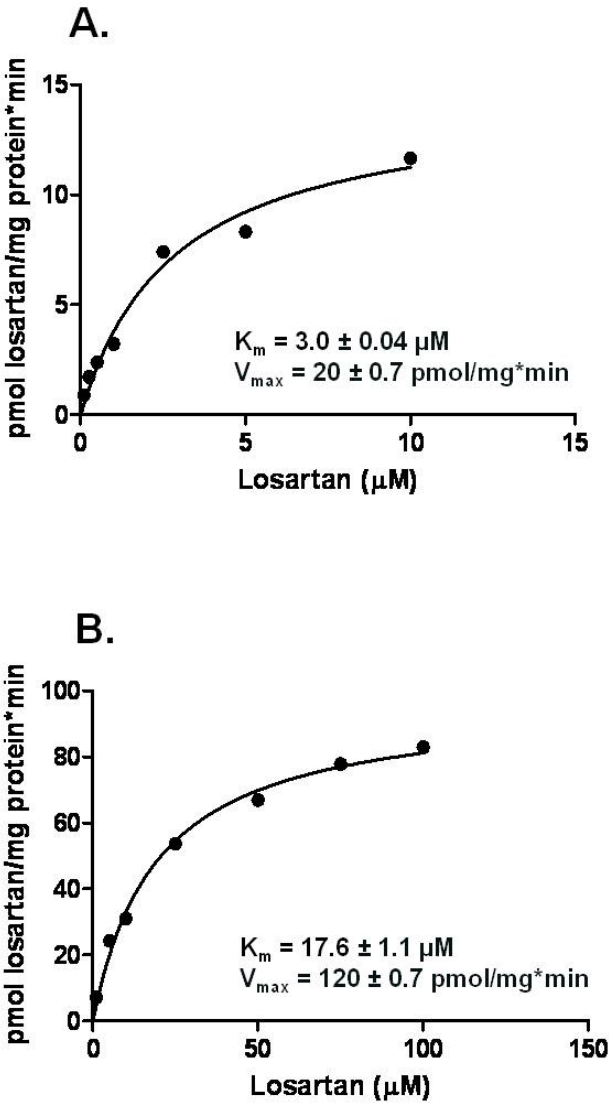


Figure 5.5: Concentration-Dependent Losartan Uptake by OATP1B1 and OATP1B3. Cells expressing human OATP1B1 (A) were exposed for 30 seconds to 0.1-10 μM losartan. Cells expressing human OATP1B3 (B) were exposed for 30 seconds to 1-100 μM losartan. Michaelis-Menten curve fitting was used to determine the K_m values of 3.0 μM for OATP1B1 and 17.6 μM for OATP1B3. Uptake was corrected for empty vector and normalized to protein. Graphs representative of one assay, average of 3 wells ± SD; K_m value representative of 3 assays ± SEM.

5.4: Discussion

Telmisartan is quite lipophilic (Strohmenger et al., 1998) and has been shown to be transported by OATP1B3 (Yamada et al., 2007), olemesartan is administered as a pro-drug (Nakagomi-Hagihara et al., 2006) that is rapidly metabolized in the small intestine, and little is known about how valsartan is absorbed after a single dose. Since losartan is dosed orally and the most structurally similar to valsartan, we hypothesize that losartan utilizes OATPs present in enterocytes to cross the gut wall and enter portal circulation prior to metabolism in the liver. In this study we demonstrated that losartan can inhibit transport of estrone-3-sulfate and estradiol-17β glucuronide in a concentration dependent manner, and that OATP1A2, OATP2B1, OATP1B1 and OATP1B3 are all capable of transporting losartan in both a time- and concentration-dependent manner.

Inhibition of model substrate transport was an indication that losartan may be transported by various OATPs involved in absorptive processes in the small intestine and liver. Significant inhibition of estrone-3-sulfate transport was seen at both concentrations of losartan for OATP1A2 and OATP2B1 (Chapter 4), however, only at 100 μM losartan did we see inhibition of OATP1B1- or OATP1B3-mediated transport of estrone-3-sulfate (Figure 5.1.1 and 5.1.2). This variation in inhibition may be due to differing substrate affinities for OATP1B1 and OATP1B3. Therefore we also tested if an

additional substrate, estradiol-17β-glucuronide, could be modulated by losartan and observed significant concentration dependent inhibition by losartan at 10 and 100 μM.

Since OATP1A2 and 2B1 are capable of transporting losartan in a time and concentration-dependent manner (Figure 5.3), it seems likely that these two OATPs play a role in losartan absorption and disposition in the gut. As previously mentioned, other angiotensin II receptor antagonists are substrates of OATP1B1 and 1B3 and they also share structural similarity with losartan. Since losartan is metabolized to E3174 in the liver prior to exerting its anti-hypertensive effects, we hypothesized that OATP1B1 and/or OATP1B3 would be capable of transporting losartan into hepatocytes. We were then able to show that losartan is transported by OATP1B1 and OATP1B3 in a time and concentration-dependent manner (Figure 5.4) and plays a role in the uptake of losartan into the liver for metabolism by CYP2C9.

Based on the Michaelis constants calculated here, OATP2B1 (K_m = 16.6 μM) has a greater affinity for losartan than OATP1A2 (K_m = 58.2 μM) and OATP1B1 (K_m = 3.0 μM) has a greater affinity for losartan than OATP1B3 (K_m = 17.6 μM). This implies that if drug-drug interactions involving OATP transport of losartan were to occur, it would more likely be at the OATPs with the lesser affinity for losartan. Those substrates with larger K_m values and therefore less affinity for the substrate bind the substrate to a lesser degree and can be sites of drug-drug interactions. Therefore OATP1A2 and/or OATP1B3 would be sites for drug-drug interactions based on lesser affinities for

losartan. However, since substrate specificity varies between OATPs as well as overlaps, assessing drug-drug interactions with losartan may require multiple substrates.

The inability to saturate OATP2B1 at pH 5.5 appears to be a direct consequence of the chemistry of losartan, and the tetrazole ring in particular. The tetrazole ring on losartan has a pK_a of 4.89, leaving it neutrally protonated at pH 7.4. Decreasing the pH to 5.5 in order to broaden OATP2B1 substrate specificity increases the amount of uncharged losartan present in the system. At pH 5.5 80-times more losartan is uncharged and can freely diffuse across the cell membrane as compared to pH 7.4. This can be better illustrated in Figure 5.2.2, where empty vector expressing cells of OATP2B1 (A) contain approximately 10 pmol losartan at pH 7.4, whereas empty vector expressing cells of OATP2B1 at pH 5.5 contain 50 times more losartan at pH 5.5 (B).

In conclusion, the transport of losartan by major OATPs expressed in the enterocyte and in the hepatocyte suggests that these proteins may play a significant role in losartan disposition, by playing roles in its uptake from the gut (absorption) and uptake into the liver (distribution) for metabolism. The multi-specific, overlapping substrate specificity of OATPs and the ability of losartan to be transported by multiple OATPs in both the enterocyte and hepatocyte has direct implications for drug-drug interactions involving this probe drug.

Chapter 6

OATP1A2 Single Nucleotide Polymorphisms and Their Effect on Fexofenadine Transport and Drug-Drug Interactions

6.1: Abstract

OATP1A2 is capable of transporting the H_1-receptor antagonist fexofenadine in both a time and concentration dependent manner. Because OATP1A2 is multi-specific and capable of transporting a wide range of drugs, xenobiotics, and endogenous compounds, either drug-drug or drug-diet interactions may alter its function. Genetic polymorphisms also can alter transporter function. We have observed fexofenadine-drug interactions *in vivo* and could explain them by OATP1A2 inhibition *in vitro* (Chapter 4). We observed that buspirone, dextromethorphan, and losartan, components of the Kansas Cocktail, are capable of inhibiting fexofenadine transport by OATP1A2. To identify whether or not OATP1A2 single nucleotide polymorphisms could play a role in the fexofenadine-drug interaction seen *in vivo*, we performed site-directed mutagenesis and created three OATP1A2 mutants (Ile13Thr, Arg168Cys, and Glu172Asp) known to alter substrate transport. To determine the effects of each mutation on OATP1A2 function and drug-drug interactions, we expressed these mutants in HEK293 cells and measured uptake of 30 μM fexofenadine for 2 minutes alone and in the presence of low and high concentrations of buspirone, dextromethorphan, and losartan. While the mutants functioned as expected with regards to fexofenadine transport, the presence of

the mutation did not alter the observed drug-drug interactions seen previously with OATP1A2 and components of the Kansas Cocktail. Together these data suggest that the fexofenadine-drug interaction seen *in vivo* is likely a result of a direct drug-drug interaction and not due to a single nucleotide polymorphism.

6.2: Introduction

The absorption of drugs dosed orally depends on passive diffusion or carrier mediated transport through cell membranes. Organic anion transporting polypeptides (OATPs) are a family of multi specific membrane uptake transporters capable of transporting a wide range of drugs, xenobiotics, and endogenous compounds into cells (Hagenbuch and Meier, 2004). Organic anion transporting polypeptide 1A2 (OATP1A2) is primarily expressed in epithelial cells of the kidney, bile ducts, intestine, and in endothelial cells of the blood-brain barrier (Gao et al., 2000; Su et al., 2004; Lee et al., 2005). Because OATP1A2 is known to be a drug uptake transporter with broad substrate specificity that includes many clinically used drugs it likely plays an important role in tissue-specific disposition, pharmacokinetics, and toxicity of xenobiotics. Therefore, genetic variations in *SLCO1A2* may alter transport kinetics or drug-drug and drug-diet interactions, and thus may have important implications in regard to the disposition of drugs.

Direct effects of genetic variants of OATP1A2 on transporter function have been described. Lee et al. (2005) identified 6 non-synonymous single polymorphisms (SNPs) within the coding region of *SLCO1A2* (Ile13Thr, Asn128Tyr, Asn135Ile, Glu172Asp, Ala178Thr, and Thr668S). *In vitro* functional studies revealed that the Asn135Ile and Glu172Asp variants had reduced uptake capacity for estrone-3-sulfate, deltorphin II, and [D-penicillamine2,5]-enkephalin. Other variants had substrate specific altered transport activity. Kinetic analysis of the transport data indicated that mutations at amino acids 128, 135, 172, 187, and 668 have functional consequences (Lee et al., 2005).

Badagnani et al. (2006) described seven protein-altering OATP1A2 variants found in 270 ethnically diverse samples. Four of the seven variants (Ile13Thr, Arg168Cys, Glu172Asp, and Thr668Ser) were found to exhibit altered transport of estrone-3-sulfate or methotrexate. Because these genetic variants exhibit altered transport properties, we wondered whether these variants also would alter drug-drug interactions at OATP1A2. We chose to examine this possibility using fexofenadine as the substrate and constructed the OATP1A2 mutants Ile13Thr, Arg168Cys, and Glu172Asp. In order to compare the effect of probe dugs on the function of wild-type or mutant OATP1A2, wild-type OATP1A2 and the three variantes were expressed in HEK293 cells and uptake of fexofenadine was determined in the absence and presence of the CYP probe drugs buspirone, dextromethorphan, and losartan.

6.3 Results:

6.3.1 Alterations in Fexofenadine Uptake by OATP1A2 Variants.

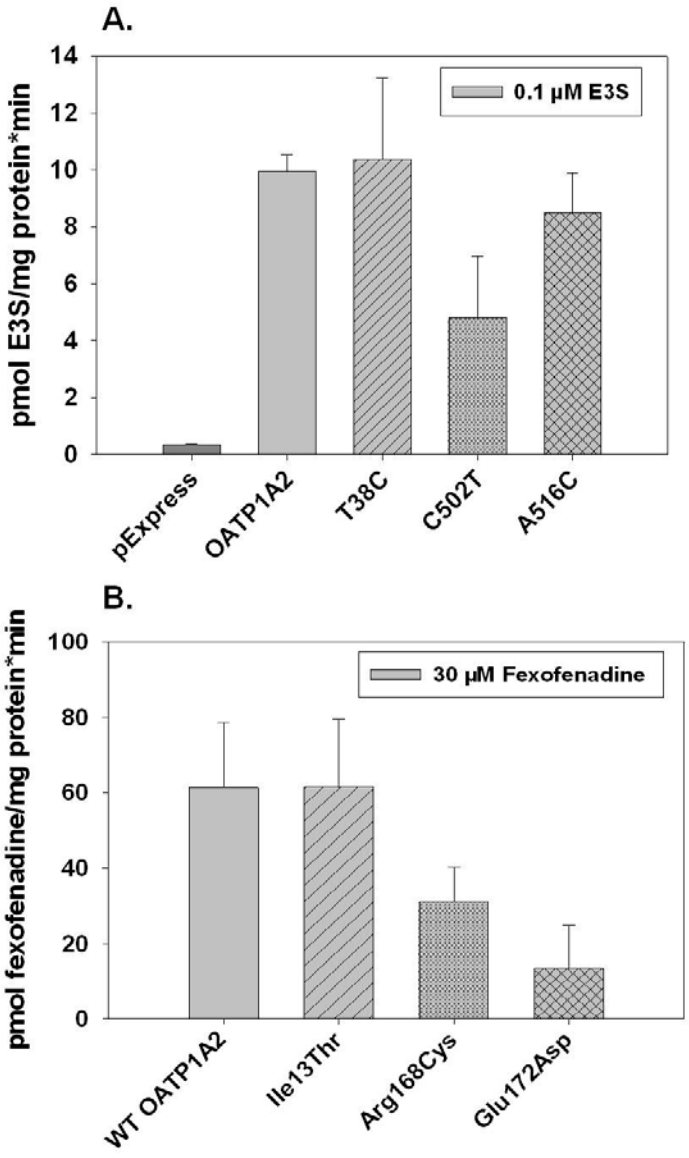
It has been shown that OATP1A2 variants with non-synonymous single nucleotide polymorphisms have altered transport of estrone-3-sulfate and methotrexate (Lee et al., 2005; Badagnani et al., 2006). We decided to test three of these OATP1A2 variants, Ile13Thr, Arg168Cys, and Glu172Asp, for their ability to transport 0.1 μM ^{3}H-estrone 3-sulfate (A) and 30 μM fexofenadine (B) as compared to wild-type OATP1A2. Figure 6.1 illustrates that Ile13Thr transported both substrates to the same extent as wild-type OATP1A2. Arg168Cys transported both substrates to only about 50% of the rate obtained with wild-type OATP1A2 while Glu172Asp showed a substrate specific effect. It had similar estrone-3-sulfate uptake as wild-type OATP1A2 but uptake of fexofenadine was reduced by 80% when compared to wild-type control.

6.3.2 Fexofenadine-Losartan Drug-Drug Interactions and OATP1A2 Variants.

As shown in Chapter 4, buspirone, dextromethorphan and losartan, three components of the Kansas Cocktail, were capable of inhibiting fexofenadine transport by OATP1A2 *in vitro*. Figure 6.2 demonstrates that losartan was able to inhibit transport of 30 μM fexofenadine by wild-type OATP1A2 (A) as well as by the three variants at

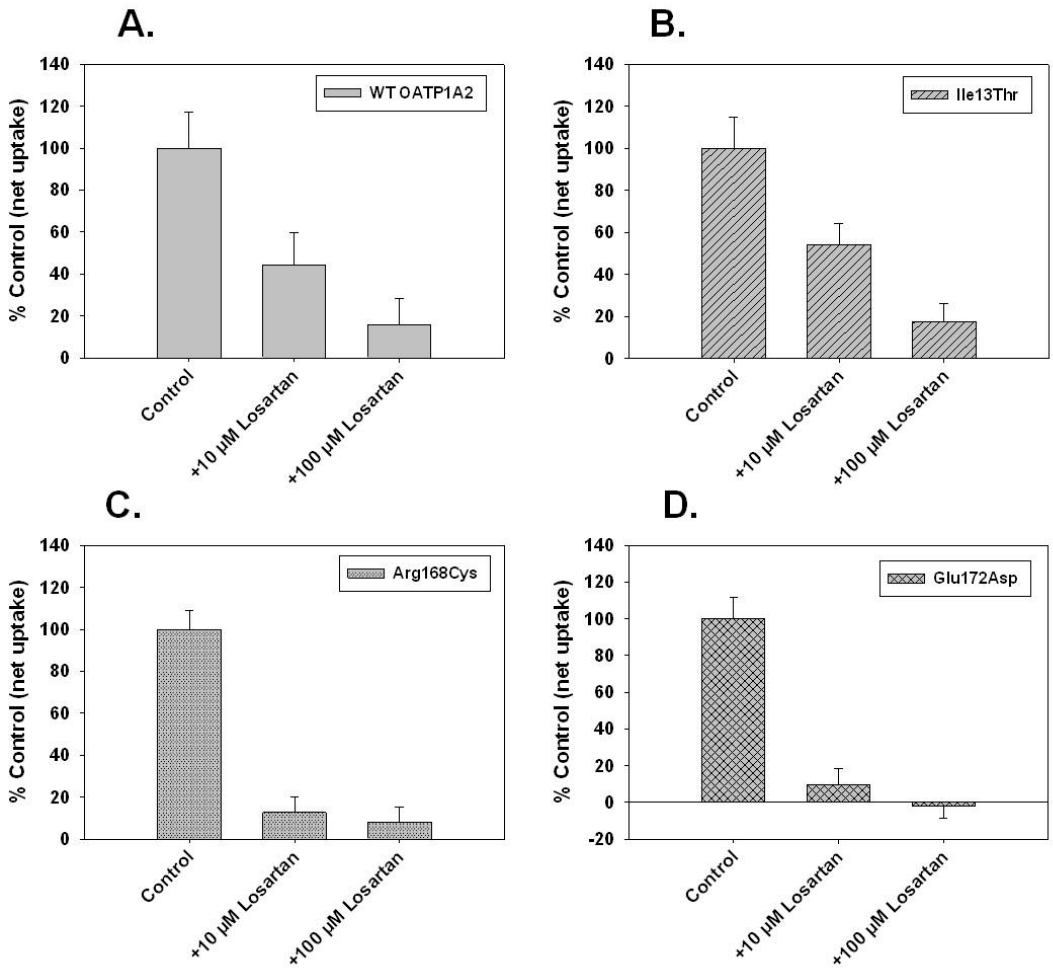
both 10 and 100 μM concentrations of losartan. The different OATP1A2 variants were inhibited to at least the same degree as wild-type OATP1A2, including Ile13Thr (B). Inhibition of Arg168Cys- and Glu172Asp-mediated fexofenadine transport (C and D) was even more complete than inhibition of wild-type OATP1A2 probably due to the fact that fexofenadine uptake by these two variants was reduced overall. Taken together, the introduction of OATP1A2 mutations does not seem to alter the fexofenadine-losartan interaction seen previously.

Figure 6.1:



Alterations in Substrate Uptake by OATP1A2 Variants. HEK293 cells transiently expressing OATP1A2 were incubated with 0.1 µM ^{3}H-estrone-3-sulfate (A) for 5 minutes or 30 µM fexofenadine (B) for 2 minutes at 37°C. After correction for protein, uptake into empty vector cells was subtracted to obtain net uptake. Each value is the mean ± SEM of three independent experiments.

Figure 6.2:

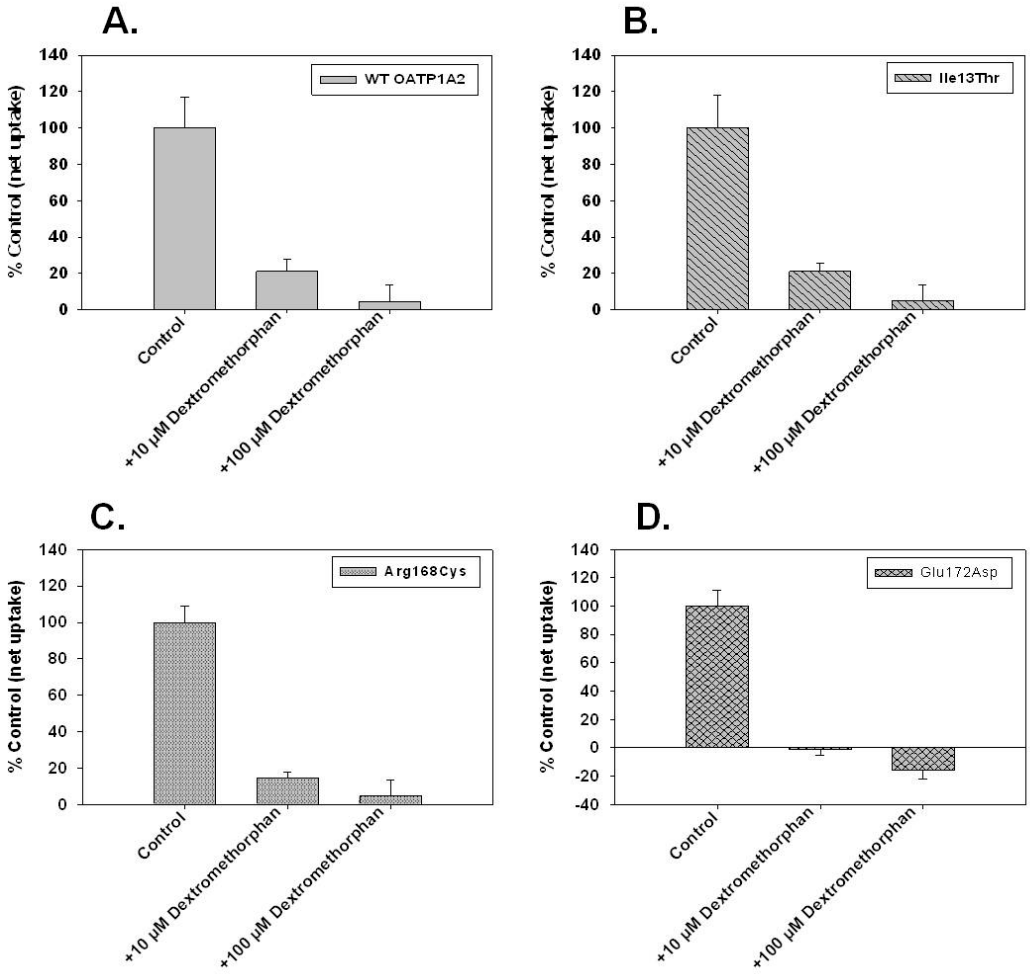


Fexofenadine-losartan drug-drug interactions with OATP1A2 variants. HEK293 cells transiently expressing WT OATP1A2 (A), Ile13Thr variant (B), Arg168Cys (C), and Glu172Asp (D) were incubated with 30 µM fexofenadine alone (control) or in the presence of 10 or 100 µM losartan for 2 minutes at 37°C. After normalizing to protein and subtracting the values obtained in cells expressing the empty vector, uptake of fexofenadine was expressed as a percentage of wild-type. Each value is the mean ± SEM of three independent experiments.

6.3.3 Fexofenadine-Dextromethorphan Drug-Drug Interactions and OATP1A2 Variants.

Dextromethorphan, a CYP2D6 probe, was capable of significant inhibition of 30 μM fexofenadine transport by WT OATP1A2 at 10 and 100 μM concentrations of losartan (Figure 6.3, A). The hyperfunctional Ile13Thr variant does not alter the inhibition of fexofenadine transport by dextromethorphan in comparison to WT OATP1A2 expressing cells (B). Hypofunctional variants Arg168Cys and Glu172Asp (C and D) transport less fexofenadine overall, therefore their percent inhibition by 10 and 100 μM dextromethorphan appears more significant because these mutants transport less fexofenadine overall. Taken together, the introduction of OATP1A2 mutations does not seem to alter the fexofenadine-dextromethorphan interaction seen previously.

Figure 6.3:



Fexofenadine-dextromethorphan drug-drug interactions with OATP1A2 variants. HEK293 cells transiently expressing WT OATP1A2 (A), Ile13Thr variant (B), Arg168Cys (C), and Glu172Asp (D) were incubated with 30 µM fexofenadine alone (control) or in the presence of 10 or 100 µM dextromethorphan for 2 minutes at 37°C. After normalizing to protein and subtracting the values obtained in cells expressing the empty vector, uptake of fexofenadine was expressed as a percentage of wild-type. Each value is the mean ± SEM of three independent experiments.

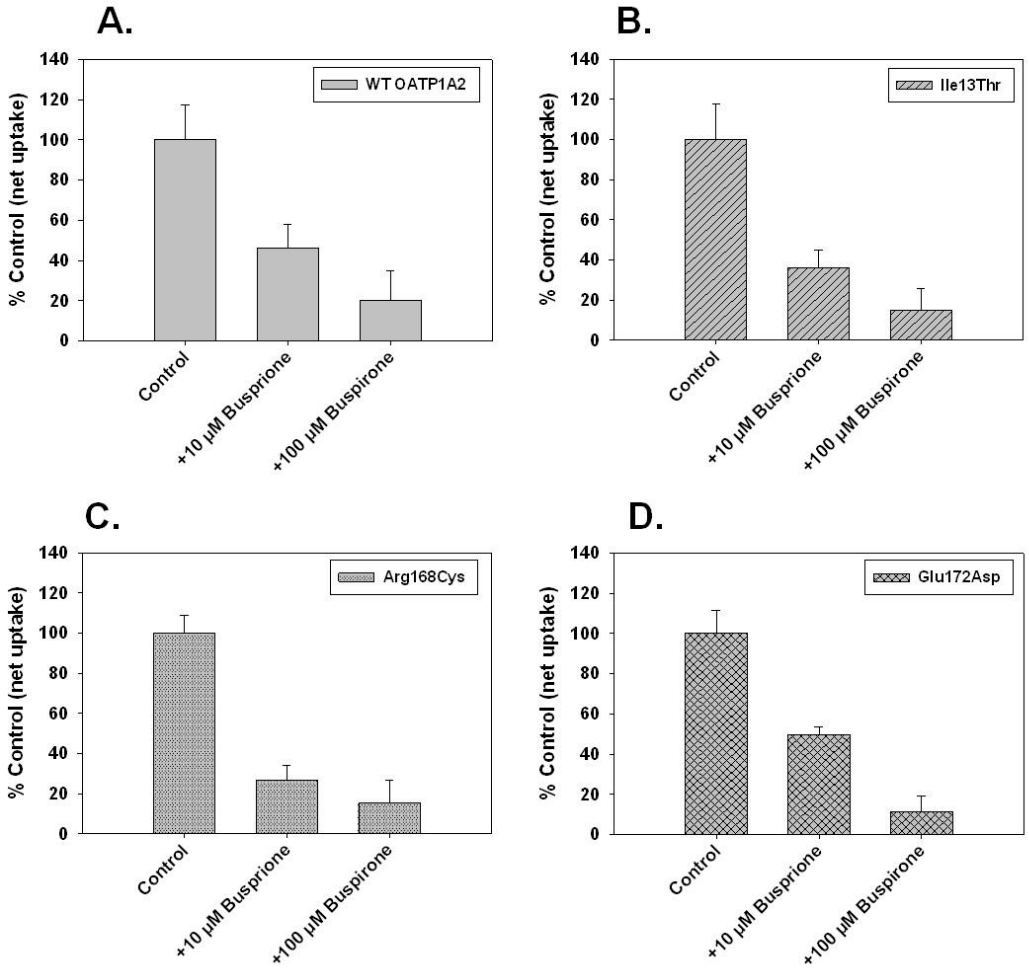
6.3.4 Fexofenadine-Buspirone Drug-Drug Interactions and OATP1A2 Variants.

Buspirone, a CYP3A4 probe, was capable of significant inhibition of 30 μM fexofenadine transport by WT OATP1A2 at 10 and 100 μM concentrations of losartan (Figure 6.4, A). The hyperfunctional Ile13Thr SNP does not alter the inhibition of fexofenadine transport by busprione in comparison to WT OATP1A2 expressing cells (B). Hypofunctional variants Arg168Cys and Glu172Asp (C and D) transport less fexofenadine overall, therefore their percent inhibition by 10 and 100 μM buspirone appears more significant because these mutants transport less fexofenadine overall. Taken together, the introduction of OATP1A2 mutations does not seem to alter the fexofenadine-busprione interaction seen previously.

6.4: Discussion

In this study we demonstrated that three OATP1A2 variants are capable of transporting both estrone-3-sulfate and fexofenadine in an altered manner from that of wild-type OATP1A2. While we saw differences in fexofenadine transport by the OATP1A2 variants, the fexofenadine-drug interactions were not significantly affected by the presence of a single nucleotide polymorphism, indicating that the fexofenadine-drug interaction seen in Chapter 4 is not due to genetic variation, but the interaction between one or more probe drugs and fexofenadine at OATP1A2.

Figure 6.4:



Fexofenadine-buspirone drug-drug interactions with OATP1A2 variants. HEK293 cells transiently expressing WT OATP1A2 (A), Ile13Thr variant (B), Arg168Cys (C), and Glu172Asp (D) were incubated with 30 µM fexofenadine alone (control) or in the presence of 10 or 100 µM busprione for 2 minutes at 37°C. After normalizing to protein and subtracting the values obtained in cells expressing the empty vector, uptake of fexofenadine was expressed as a percentage of wild-type. Each value is the mean ± SEM of three independent experiments.

Site directed mutagenesis allowed us to create three OATP1A2 variants seen previously in human subjects, Ile13Thr, Arg168Cys, and Glu172Asp (Lee et al., 2005; Badagnani et al., 2006) and examine the effects of these mutations on the transport of fexofenadine. We saw quantitative similarities in how all three variants transported estrone-3-sulfate when compared to results by Badagnani et al (2006), with the Ile13Thr mutant being hyperfunctional and the Arg168Cys mutant showing hypofunctionality with transport of 0.1 μM estrone-3-sulfate. There were no observed changes in V_{max} (as reported by Badagnani et al.) associated with Glu172Asp; however, since we only examined one concentration of estrone-3-sulfate, we would not expect to see changes in capacity of transport (Figure 6.1, A). When examining transport of fexofenadine by OATP1A2 variants, there was no difference in transport by Ile13Thr when compared to wild-type OATP1A2 and both Arg168Cys and Glu172Asp demonstrate hypofunctionality in transport (Figure 6.1, B). This suggests that while each OATP1A2 mutant is capable of altering substrate transport, that the degree of transport is somewhat substrate specific.

While we saw differences in fexofenadine transport by OATP1A2 variants, the fexofenadine-drug interactions were not significantly affected by the presence of a single nucleotide polymorphism. Losartan was capable of inhibiting transport of fexofenadine by WT OATP1A2, as well as Ile13Thr, Arg168Cys, and Glu172Asp; however, there was no enhanced inhibition or stimulation of uptake in the presence of these mutations (Figure 6.2). Since variants Arg168Cys and Glu172Asp have reduced transport of fexofenadine, the degree of inhibition appears greater than wild-type, yet

the phenomenon observed is less fexofenadine transported, but the same percentage of inhibition by losartan. Similar trends can also be seen for dextromethorphan-fexofenadine interactions (Figure 6.3) and buspirone-fexofenadine interactions (Figure 6.4). While each OATP1A2 variant has different affinity or capacity for fexofenadine, it the fexofenadine-drug interaction is not altered by the presence of a single nucleotide polymorphism.

Genotypic analysis of the OATP1A2 variants showed that allelic frequency was dependent on ethnicity. The hyperfunctional mutant (Ile13Thr) examined by both Lee et al. (2005) and Badagnani et al (2006) is present in European-American populations at 11.1% and 16.3% respectively. The novel hypofunctional Arg168Cys mutant is present in 0.6% of European Americans sampled, and the Glu172Asp hypofunctional mutant is present in European American populations at 5.3% and 1.9%, respectively (Lee et al., 2005; Badagnani et al., 2006). In examining the allelic frequencies of these mutations and their lack of effect in our *in vitro* fexofenadine-drug interaction studies, it is highly unlikely that the majority of our sample population falls into the ethnic groups with the most diversity and alteration of transport.

In summary, our studies suggest that protein-altering variants in OATP1A2 do not play a role in inter-individual variability of fexofenadine-probe drug interactions. While transport of fexofenadine as a substrate is altered by three known variants of OATP1A2 (Ile13Thr, Arg168Cys, and Glu172Asp), they do not contribute significantly to

fexofenadine transport inhibition by losartan, dextromethorphan, or buspirone. Decreases in AUC and C_{max} of fexofenadine when dosed as part of the Kansas Cocktail are likely to occur from a direct drug-drug interaction at OATP1A2 versus a genetic polymorphism. Differing expression levels of WT OATP1A2 or variation in fexofenadine bioavailabilty between human subjects may potentially play a role in the fexofenadine-drug interaction observed previously, but not the three SNPs examined here.

Chapter 7

Summary and Discussion

7.1: Significance

Inter- and intra-individual variability in responses to drugs poses a major clinical challenge that needs to be managed, often arising from differences in pharmacokinetics of the drug. Understanding the cause, being able to predict changes, and managing variation in pharmacokinetic responses requires the utilization of better experimental approaches and investigation of mechanisms responsible for these phenomena. Probe drugs represent a potentially valuable approach to the study of variability in pharmacokinetics; however, there are few specific probe drugs for Phase 2 enzymes or for transporters that have been well characterized. Membrane transporters are proteins that transport drugs across cellular membranes and play an integral role in the absorption, distribution, metabolism, and excretion of drugs. Due to their important role in the disposition of drugs, changes in transport activity ultimately result in alteration of the pharmacokinetics of drugs, thereby impacting their effects.

Active and facilitative drug transporting molecules, like P-glycoprotein and organic anion transporting polypeptides (OATPs), play an important role in the absorption, distribution, metabolism, and excretion of drugs. P-glycoprotein is a

membrane transport efflux protein that is highly expressed throughout the body and contributes to the decreases in both the absorption and the distribution of drugs to organs, tissues, and cells. Opposing P-glycoprotein is the OATP family of transporters, which act as uptake transporters and facilitate absorption and distribution of endogenous hormones, drugs, and xenobiotics throughout the body. Of particular interest, OATP1A2 is expressed in many tissues including the apical membrane of the small intestine, blood brain barrier, kidney, and cholangiocytes of the liver.

After a drug is given orally, it must move through several biological membranes to exert its pharmacological effect. Since OATPs are localized to cell membranes and actively move drugs through such barriers, they play a role in pre-systemic drug-drug interactions and first-pass effects. Polymorphisms in OATPs have been associated with altered pharmacokinetics of many substrates, including methotrexate, deltorphin, and estrone-3-sulfate, demonstrating their significance to drug disposition. Many drug-drug and drug-food interactions at OATPs can also alter the pharmacokinetic profiles of many drug substrates, leading to decreased efficacy or increased toxicity. Inclusion of fexofenadine as a probe for transport activity in the Kansas Cocktail resulted in a previously unreported fexofenadine-drug interaction. Since fexofenadine is minimally metabolized, its kinetics are controlled by transporters; however, there is still uncertainty in regards to which membrane transporters are controlling fexofenadine pharmacokinetics and to what extent.

In this dissertation, the studies presented were designed to better elucidate the OATPs involved in fexofenadine pharmacokinetics, as well as define the exact nature of the fexofenadine-drug interaction seen clinically with the Kansas Cocktail. To do this, I formulated my central hypothesis around fexofenadine being primarily transported by OATP1A2 and that the fexofenadine-drug interaction was occurring at this transporter. This hypothesis was then tested via four specific aims: first, development of a sensitive, rapid, and specific method for quantifying fexofenadine from cell culture lysates; second, definition and characterization of the kinetics of fexofenadine with human OATP uptake transporters known to be expressed in the enterocyte and the hepatocyte; third, identification of probe drug(s) interacting with fexofenadine at defined transporter(s); and fourth, exploration of the effect of OATP1A2 variants on fexofenadine transport kinetics and fexofenadine-probe drug interactions.

7.2: Specific Aim 1

In my first Specific Aim, I developed and fully validated a high performance liquid chromatography-tandem mass spectrometry method for quantification of fexofenadine in mammalian cell lysates, with cetirizine used as the internal standard. Using the FDA Guidelines for Bioanalysis (FDA, 2001) as a guide, we determined the linearity, accuracy, precision, and recovery of fexofenadine in both mobile phase and HEK293 cell lysates. This method was then applied to the analysis of fexofenadine in cell

lysates from *in vitro* transporter studies, and further developed to measure other probe drugs to support drug-drug interaction studies in these model systems.

This study showed that the most sensitive transitions were 502.17/466.2 for fexofenadine and 389.09/201.1 for the internal standard cetirizine. We optimized chromatographic conditions by testing a variety of aqueous and organic mobile phases. Based on sensitivity, peak shape for the analyte and internal standard, and on overall run time, we chose 7.5 mM ammonium formate, pH 5 as an aqueous mobile phase and 50/50 (v/v) acetonitrile:methanol mixture for the organic component. Use of a gradient system allowed elution of fexofenadine at 2.48 minutes and the internal standard at 2.66 minutes with a total run time of less than five minutes.

Method selectivity was examined by analyzing blank cell culture matrix samples and matrix spiked only with internal standard. There was no endogenous matrix interference at the retention times of the fexofenadine or cetirizine. Similarly, the cetirizine (IS) does not show any crossover in the MRM of fexofenadine. The assay was sensitive for fexofenadine at concentrations as low as 1 ng/mL, which was also the lower limit of quantitation (LLOQ) of fexofenadine. The calibration curve for fexofenadine extracted from HEK293 cell lysates was linear over the concentration range of 1-500 ng/mL fexofenadine, with an r^2 of 0.99.

Fexofenadine quantitation at 1 ng/mL was accomplished with a precision of 2.6% and a mean accuracy of 106.5%, well within the limits for defining the LLOQ. Similarly,

the precision and accuracy of each concentration of fexofenadine in the calibration curve met the FDA criteria for bioanalytical method validation. The inter-day coefficient of variation (CV) for the lowest QC standard (3 ng/mL) in cell lysate is 9.5% and the between batch accuracy was 97.4%. Within the batches, the low QC standard had a CV of 8.5% and the accuracy was 91.9%. For the middle and upper quantification levels, ranging from 75-500 ng/mL, the precision ranged from 2.5-6.2% and the accuracy from 90.1-97.0% within batch. Between batches, the precision ranged from 3.4-4.9% and the accuracy from 97.4-103.6% in HEK293 cell lysate. As a final experiment, I looked at recovery of fexofenadine from cell lysates as compared to blank matrix. The average recovery of fexofenadine was from 86% to 123% and with the exception of the LLOQ; all concentrations had standard deviations of recovery less than 20%.

My first specific aim clearly demonstrates that we have developed a highly sensitive and specific assay for the quantification of fexofenadine in cell lysates. In order to use fexofenadine as a probe of transporter function and measure fexofenadine transporter activity, a rapid, specific, and sensitive analytical method has been developed to meet this need. Compared to other analytical methods, including HPLC with fluorescence detection and existing LC-MS/MS methods, this aim was able to provide improved selectivity and sensitivity. Our method yields a LLOQ of 1 ng/mL, versus previously published LLOQs of greater than 1 ng/mL. We were also able to expand the method to include other probe drugs, including buspirone, caffeine, dextromethorphan, and losartan. Utilization of the same gradient and mobile phases, sample preparation procedure, and internal standard, as well as a run time under 5

minutes, made this method ideal for measuring the effects of the Kansas Cocktail on transporter activity.

7.3: Specific Aim 2

In the first Specific Aim, we addressed a need to develop a specific and sensitive method for measuring fexofenadine in HEK293 cell culture lysates from transporter experiments. In the second Specific Aim, we wanted to define and characterize the kinetics of fexofenadine with OATP uptake transporters known to be expressed in the enterocyte and the hepatocyte. The transporters studied were OATP1A2, 1B1, 1B3, and 2B1. We then utilized the analytical method developed in Specific Aim 1 to measure fexofenadine uptake in these systems.

To test whether fexofenadine was a substrate of OATPs expressed in the small intestine (OATP1A2, OATP2B1) or in the liver (OATP1B1, OATP1B3), I first used three concentrations of fexofenadine (1, 10, 100 μM) over a 10 minute time period to see if any OATP-dependent uptake of fexofenadine was observed. OATP1A2 transported 50-150 times more fexofenadine than OATP2B1 at all concentrations tested; and while uptake seemed to increase with concentration for OATP2B1, the absolute level was too low to determine kinetic parameters. This result suggested that OATP2B1 may not be a major contributor to fexofenadine uptake system in enterocytes. However, we were

able to characterize the effective fexofenadine transport by OATP1A2 in both time and concentration dependent manners, with a K_m of 35 μM. These findings support similar observations by Cvetkovic et al. (1999), Glaeser et al. (2007), and Shimizu (2005). It is important to note however, that if expression levels of OATP2B1 were significantly greater than those of OATP1A2, transport of fexofenadine by OATP2B1 might play a more significant role in the disposition of fexofenadine in the small intestine than our results would indicate. Based on the experiments presented in this dissertation and the central hypothesis of this project, OATP1A2 is a major transporter of fexofenadine and may play a dominant role in its disposition.

Similarly, to test fexofenadine uptake by OATP1B1 and OATP1B3, I first used three concentrations of fexofenadine (1, 10, 100 μM) over a 10 minute time period to see if any liver specific OATP-dependent uptake of fexofenadine was observed. OATP1B1 was capable of fexofenadine transport, as was OATP1B3, however, OATP1B3 transported at least three times more fexofenadine at all concentrations tested. Since fexofenadine was dosed orally as part of the Kansas Cocktail and our central hypothesis assumes the fexofenadine-drug interaction is occurring at an OATP in the small intestine, we did not explore OATP1B1, OATP1B3 and fexofenadine transport by hepatocytes any further in this dissertation.

7.4: Specific Aim 3

Based on observations in a previous clinical study, a decrease in fexofenadine C_{max} and AUC occurs when fexofenadine is dosed with the CYP probes buspirone, caffeine, dextromethorphan, fexofenadine, losartan as part of the Kansas Cocktail. In Specific Aim 2, we characterized the kinetics of fexofenadine transport by OATP1A2 and acknowledged its importance in the disposition of orally dosed fexofenadine. In Specific Aim 3, the goal was to characterize which of the probe drugs interacted with fexofenadine at OATP1A2. Recapitulation of the fexofenadine-drug interaction seen in human subjects in this cell-based model system provides further support for the identity of the dominant uptake transporter controlling fexofenadine pharmacokinetics.

An initial screen of OATP1A2-expressing HEK293 cells showed that fexofenadine, buspirone, dextromethorphan, and losartan were each capable of inhibiting transport of 0.1 μM estrone-3-sulfate in a concentration dependent manner. However, only losartan inhibited transport of 0.1 μM estrone-3-sulfate transport by OATP2B1. Modulation of model substrate transport allowed us to elucidate which probe drugs may be inhibiting fexofenadine transport in the small intestine and explain our *in vivo* observations with fexofenadine and the Kansas Cocktail.

Additional studies demonstrated that losartan, dextromethorphan, and buspirone each inhibited transport of fexofenadine by OATP1A2 in a concentration-dependent manner. The efficient transport of fexofenadine by OATP1A2 suggests that the activity

of this transporter may be a major determinant of the pharmacokinetics of orally dosed fexofenadine. Furthermore, the inhibition of OATP1A2-dependent transport of fexofenadine by three of the drugs in the Kansas Cocktail is consistent with the decrease in fexofenadine bioavailability seen in the clinical study. Net fexofenadine transport and the replication of the fexofenadine-probe drug interaction in this model system supports the conclusion in Specific Aim 3 that OATP1A2 is the major uptake transporter for fexofenadine absorption, and suggests that fexofenadine interacts with multiple probe drugs.

Based on the fexofenadine-CYP probe drug interactions we have characterized, the use of fexofenadine as a probe drug in conjunction with these probes for CYP activity is not recommended. OATPs have wide, overlapping substrate specificity and are not inhibited or stimulated by any one endogenous compound, xenobiotic, or pharmaceutical drug. Fexofenadine itself is a substrate of multiple OATPs and P-glycoprotein, as well as being a part of numerous drug-drug and drug-diet interactions outside the clinical interaction presented as part of this dissertation. Inclusion of fexofenadine in a probe drug cocktail would require validation that there were no significant pharmacokinetic interactions between each CYP probe and fexofenadine, since this would result in fexofenadine kinetics that were already altered and thus would greatly complicate the assessment of the affects of additional drugs or dietary factors on transporter function..

Interestingly, when we did the screens for modulation of model substrate activity by the probe drug cocktail with estrone-3-sulfate and OATP1A2 and OATP2B1, we also included screens for OATP1B1 and OATP1B3 as well as inclusion of a second substrate (estradiol-17β-glucuronide). When examining the effects of each probe drug on transport of estrone-3-sulfate or estradiol-17β-glucuronide by each OATP, only losartan at 10 or 100 μM was capable of significant inhibition of model substrate transport. This indicated that losartan had the potential to be transported by multiple enterocyte- and hepatocyte-specific OATPs that may play a role in the disposition of this orally dosed drug.

Based on these observations, we hypothesized that both enterocyte and hepatocyte-specific OATPs are involved in the uptake and disposition of losartan. We tested this hypothesis by characterizing losartan uptake using OATP1A2, OATP2B1, OATP1B1, and OATP1B3 expressing cell lines and determined the kinetics of losartan transport by 4 OATPs. For OATP1A2, a K_m of 58 μM was determined for losartan uptake and a K_m of 16.6 μM for OATP2B1-expressing cells. Based on these values, it appears that OATP2B1 has a greater affinity for losartan and may be responsible for uptake of orally dosed losartan in enterocytes. OATP1B1 has a K_m of 3 μM for uptake of losartan, with OATP1B3 having a K_m of 17.6 μM for losartan. In the hepatocyte, OATP1B1 appears to have a greater affinity for losartan and may be responsible for the uptake of this drug into the liver where it is metabolized by CYP2C9 into E3174. This then implies that if drug-drug interactions involving OATP transport of losartan were to occur, it would more likely be at the OATPs with the higher affinity for losartan.

Therefore OATP2B1 and/or OATP1B1 would be sites for drug-drug interactions based on greater affinity for losartan. However, since substrate specificity varies between OATPs as well as overlaps, assessing drug-drug interactions with losartan may require multiple substrates.

7.5: Specific Aim 4

In Specific Aims 2 and 3, we explored the transport of fexofenadine by various OATPs and assessed *in vitro* the effects of three probe drugs (buspirone, dextromethorphan, losartan) from the Kansas Cocktail on fexofenadine transport by OATP1A2. In Specific Aim 4, I explored the effect of OATP1A2 genotypes on fexofenadine-probe drug interactions. Using three known human single nucleotide polymorphisms of OATP1A2, I used site-directed mutagenesis to create these variants of OATP1A2 (Ile13Thr, Arg168Cys, and Glu172Asp). After verifying their sequences, a transfected HEK293 cell system was used to determine the effects of these mutations on the transport of fexofenadine via OATP1A2 as well as the effects of the other probe drugs on fexofenadine transport.

Previous observations of OATP1A2 single nucleotide polymorphisms indicate that variants of OATP1A2 have altered transport of multiple substrates, therefore we tested Ile13Thr, Arg168Cys, and Glu172Asp with regards to their ability to transport 0.1 μM 3H-estrone 3-sulfate and 30 μM fexofenadine as compared to wild-type OATP1A2. The Ile13Thr mutant was reported to be a hyperfunctional OATP1A2 with regards to

estrone-3-sulfate and MTX transport, however, in our system, we see no increase in 30 μM fexofenadine transport above that seen with wild-type OATP1A2. Both Arg168Cys and Glu172Asp mutants were reported to be hypofunctional OATP1A2 with regards to estrone-3-sulfate and MTX transport, and we found that they also exhibit reduced transport of 30 μM fexofenadine when compared to wild-type OATP1A2. Each mutant transports estrone-3-sulfate in a manner previously reported by Badagnani et al. (2006).

In Specific Aim 3, three components of the Kansas Cocktail were capable of inhibiting fexofenadine transport by WT OATP1A2. Losartan was capable of significant inhibition of 30 μM fexofenadine transport by WT OATP1A2 at 10 and 100 μM concentrations of losartan; however, introduction of the hyperfunctional Ile13Thr variant does not alter the inhibition of fexofenadine transport in comparison to WT OATP1A2 expressing cells. Hypofunctional variants Arg168Cys and Glu172Asp transport less fexofenadine overall, therefore their percent inhibition by 10 and 100 μM losartan appears greater than it is in reality is since Arg168Cys transports approximately 50% less fexofenadine than wild-type and Glu172Asp transports 75% less fexofenadine than wild-type. Taken together, the introduction of OATP1A2 mutations does not seem to alter the fexofenadine-losartan interaction seen previously. Similar phenomena are seen with dextromethorphan and buspirone; their presence inhibits fexofenadine transport by all three OATP1A2 variants, but does not significantly change the nature of the interaction when a polymorphism is present.

While the mutants functioned as intended with regards to fexofenadine transport, presence of the mutation did not alter the observed drug-drug interactions seen previously with OATP1A2 and components of the Kansas Cocktail. Taking these data into account, it appears that the fexofenadine-drug interaction seen previously is a result of a direct drug-drug interaction and not a single nucleotide polymorphism.

7.6: Future Directions

7.6.1: Allosteric Activation of P-glycoprotein

The studies presented in this dissertation demonstrate that fexofenadine is transported by OATP1A2, and more importantly that three components in the Kansas Cocktail (buspirone, dextromethorphan, and losartan) are capable of significantly inhibiting OATP1A2-mediated transport of fexofenadine. Transporters play a key role in fexofenadine disposition and the observed pre-systemic fexofenadine-drug interaction seen clinically. The inhibition of OATP1A2-dependent fexofenadine transport by three of the CYP probe drugs used in the clinical study provides a plausible mechanism for the observed interaction. The alternative hypothesis, however, is that the observed fexofenadine-drug interaction is due to allosteric activation of P-glycoprotein by a component of the Kansas Cocktail. Based on our findings reported here, it is reasonable to interpret decreases in both C_{max} and AUC of oral fexofenadine, in the

absence of changes in clearance, as a result of OATP1A2 inhibition. On the other hand, stimulation of P-gp would give the same result and such allosteric stimulation has been previously reported (Parasrampuria et al., 2001). However, P-gp inhibition is more widely documented as a drug-drug interaction (Wang et al., 2002; Dresser et al. 2003, Kamath et al. 2005, Yasui-Furukori et al. 2005, Shimizu et al., 2006) resulting in an increased C_{max} and AUC for fexofenadine.

It is important, however, to assess the role of P-glycoprotein in the efflux transport of fexofenadine and assess the impact that the CYP probes have on fexofenadine transport by P-glycoprotein. Utilizing a similar initial screening of the effects of each probe drug on P-gp efflux of a model substrate (like Rhodamine 123), we could quickly and efficiently see which probe drugs may be modulating P-gp activity. Then, using an inside out membrane vesicle system, we could measure fexofenadine transport by P-glycoprotein and test which probe drugs are capable of altering P-glycoprotein transport of fexofenadine. If any components of the Kansas Cocktail are allosterically stimulating P-gp, we should observe an increase in fexofenadine transport in the presence of probe drugs. Similarly to our single nucleotide polymorphism study with OATP1A2, we could also explore the effects of P-glycoprotein variants on transport activity of fexofenadine and whether a variant of P-glycoprotein could further enhance or inhibit transport of fexofenadine when dosed with the other Kansas Cocktail probe drugs.

7.6.2: Kansas Cocktail Human Subject Genotyping and OATP expression levels

Not all subjects showed the same fexofenadine-drug interaction when administered the full cocktail. In fact, about half of the subjects exhibited 50-75% decreases in fexofenadine Cmax and AUC from the cocktail, compared to the same dose of fexofenadine given alone, while the other half showed little effect. My fourth specific aim focused on the potential for genetic polymorphisms in OATP1A2 altering the susceptibility to these fexofenadine-probe drug interactions. The subjects from the original study could be re-consented and then genotyped for OATP1A2 to determine if the population sampled for this study matches those polymorphisms chosen. There is significant probability that the polymorphisms examined in Specific Aim 4 are not the correct variants due to not knowing the genotypes of our sample population. The ability to know and utilize each individual OATP1A2 genotype would allow us to more carefully examine the effect of inter-individual variation on the fexofenadine-drug interaction observed clinically. Studies by both Lee et al. (2005) and Badagnani et al. (2006) report novel OATP1A2 mutations when screening their clinical populations. The possibility that our subjects either overlap in genotype or present with a new single nucleotide polymorphism could explain some of the *in vivo* observations with the Kansas Cocktail.

Ideally, it would also be important to look at individual expression levels of OATPs and P-glycoprotein in our subjects. The ability to biopsy the small intestine of each subject could provide very real and relevant data on OATP1A2 and OATP2B1

expression levels in the gut. Based on our kinetic data from Specific Aim 2, we see a greater contribution of OATP1A2 to the disposition of oral fexofenadine versus that of OATP2B1 based on K_m values and fold differences in transport. However, if OATP2B1 was expressed at much higher levels, that contribution could change. Knowing the expression level of OATP1A2, OATP2B1, and even P-glycoprotein for each individual would indicate the precise location of this fexofenadine-drug interaction and give a possible indication to as to whether expression levels play a role in this observation. If single nucleotide polymorphisms do not affect the observed fexofenadine-drug interaction, variation in wild-type expression levels may alter the observed interaction, explaining why 50% of the population experiences decreased AUC, whereas the other 50% have baseline or increased AUC after dosing with the probe drug cocktail.

Further studies, like those described above, will fully elucidate the nature of the fexofenadine-drug interaction seen with the Kansas Cocktail and will clearly define the mechanism by which we see a substantial decrease in fexofenadine AUC when dosed as part of a probe drug cocktail. Understanding the nature of this interaction and the roles OATPs and P-glycoprotein play in fexofenadine transport and disposition will also define the role of fexofenadine as a potential probe drug for transporters.

References

Abduljalil K, Frank D, Gaedigk A, Klaassen T, Tomalik-Scharte D, Jetter A, Jaehde U, Kirchheiner J, and Fuhr U (2010) Assessment of activity levels for CYP2D6*1, CYP2D6*2, and CYP2D6*41 genes by population pharmacokinetics of dextromethorphan. *Clin Pharmacol Ther* 88:643-51.

Badagnani I, Castro RA, Taylor TR, Brett CM, Huang CC, Stryke D, Kawamoto M, Johns SJ, Ferrin TE, Carlson EJ, Burchard EG, and Giacomini KM (2006) Interaction of methotrexate with organic-anion transporting polypeptide 1A2 and its genetic variants. *J Pharmacol Exp Ther* **318**:521-9.

Bailey DG, Spence JD, Munoz C, and Arnold JM (1991) Interaction of citrus juices with felodipine and nifedipine. *Lancet* **337**:268-269.

Bailey DG, Dresser GK, Leake BF, and Kim RB (2007) Naringin is a major and selective clinical inhibitor of organic anion-transporting polypeptide 1A2 (OATP1A2) in grapefruit juice. *Clin Pharmacol Ther* **81**:495-502.

Bjornsson TD, Callaghan JT, Einolf HJ, Fischer V, Gan L, Grimm S, Kao J, King SP, Miwa G, Ni L, Kumar G, McLeod J, Obach SR, Roberts S, Roe A, Shah A, Snikeris F, Sullivan JT, Tweedie D, Vega JM, Walsh J, and Wrighton SA (2003) The conduct of *in vitro* and *in vivo* drug-drug interaction studies: a Pharmaceutical Research and Manufacturers of America (PhRMA) perspective. *Drug Metab Dispos* **31**:815-32.

Breimer DD and Schellens JH (1990) A 'cocktail' strategy to assess in vivo oxidative drug metabolism in humans. *Trends Pharmacol Sci* **1990**:223-5.

Capon DA, Bochner F, Kerry N, Mikus G, Danz C, and Somogyi AA (1996) The influence of CYP2D6 polymorphism and quinidine on the disposition and antitussive effect of dextromethorphan in humans. *Clin Pharmacol Ther* **60**:295-307.

Christensen M, Andersson K, Dalén P, Mirghani RA, Muirhead GJ, Nordmark A, Tybring G, Wahlberg A, Yaşar U and Bertilsson L (2003) The Karolinska cocktail for phenotyping of five human cytochrome P450 enzymes. *Clin Pharmacol Ther* **73**:517-528.

Coutant, JE, Westmark, PA, Nardella, PA, Walter, SM, and Okerholm, RA. (1991) Determination of terfenadine and terfenadine acid metabolite in plasma using solid-

phase extraction and high performance liquid chromatography with fluorescence detection. *J Chromatogr* **570**:139-146.

Cvetkovic M, Leake B, Fromm MF, Wilkinson GR and Kim RB (1999) OATP and P-glycoprotein transporters mediate the cellular uptake and excretion of fexofenadine. *Drug Metab Dispos* **27**:866-871.

Dresser GK, Bailey DG, Leake BF, Schwarz UI, Dawson PA, Freeman DJ and Kim RB (2002) Fruit juices inhibit organic anion transporting polypeptide-mediated drug uptake to decrease the oral availability of fexofenadine. *Clin Pharmacol Ther* **71**:11-20.

Dresser GK, Schwarz UI, Wilkinson GR and Kim RB (2003) Coordinate induction of both cytochrome P4503A and MDR1 by St John's wort in healthy subjects. *Clin Pharm Ther* **73**:41-50.

Dresser GK, Kim RB and Bailey DG (2005) Effect of grapefruit juice volume on the reduction of fexofenadine bioavailability: possible role of organic anion transporting polypeptides. *Clin Pharmacol Ther* **77**:170-177.

FDA Guidance for Industry: Bioanalytical Method Validation (2001) http://www.fda.gov/cder/guidance/4252fnl.pdf

FDA Guidance for Industry: In Vivo Drug Metabolism/Drug Interaction Studies – Study Design, Data Analysis, and Recommendations for Dosing and Labeling (1999) http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm072119.pdf

Flynn, CA, Alnouti, Y and Reed, GA (2011) Quantification of the transporter substrate fexofenadine in cell lysates by liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom* **25**:2361-2366.

Frye RF, Matzke GR, Adedoyin A, Porter JA and Branch RA (1997) Pharmacokinetics and drug disposition: validation of the five-drug "Pittsburgh cocktail" approach for assessment of selective regulation of drug-metabolizing enzymes. *Clin Pharmacol Ther* **62**:365-376.

Fu, I, Woolf, EJ, and Matuszewski, BK (2004) Determination of fexofenadine in human plasma using 96-well solid phase extraction and HPLC with tandem mass spectrometric detection. *J Pharmaceut Biomed* **35**:837-844.

Gaedigk A, Simon SD, Pearce RE, Bradford LD, Kennedy MJ, and Leeder JS (2008) The CYP2D6 activity score: translating genotype information into a qualitative measure of phenotype. *Clin Pharmacol Ther* **83**:234-42.

Gammans RE, Mayol RF, and LaBudde JA (1986) Metabolism and disposition of buspirone. *Am J Med* **80**:41-51.

Gao B, Hagenbuch B, Kullak-Ublick GA, Benke D, Aguzzi A, and Meier PJ (2000) Organic anion-transporting polypeptides mediate transport of opioid peptides across blood-brain barrier. *J Pharmacol Exp Ther* **294**:73-9.

Giacomini KM, Huang SM, Tweedie DJ, Benet LZ, Brouwer KL, Chu X, Dahlin A, Evers R, Fischer V, Hillgren KM, Hoffmaster KA, Ishikawa T, Keppler D, Kim RB, Lee CA, Niemi M, Polli JW, Sugiyama Y, Swaan PW, Ware JA, Wright SH, Yee SW, Zamek-Gliszczynski MJ, and Zhang L (2010) Membrane transporters in drug development. *Nat Rev Drug Discov*. **9**:215-36.

Glaeser H, Bailey DG, Dresser GK, Gregor JC, Schwarz UI, McGrath JS, Jolicoeur E, Lee W, Leake BF, Tirona RG and Kim RB (2007) Intestinal drug transporter expression and the impact of grapefruit juice in humans. *Clin Pharmacol Ther* **81**:362-70.

Hagenbuch B and Gui C (2008) Xenobiotic transporters of the human organic anion transporting polypeptides (OATP) family. *Xenobiotica*. **38**:778-801.

Hagenbuch B and Meier PJ (2004) Organic anion transporting polypeptides of the OATP/ SLC21 family: phylogenetic classification as OATP/ SLCO superfamily, new nomenclature and molecular/functional properties. *Pflugers Arch* **447**:653-665.

Hamman, MA, Bruce, MA, Haehner-Daniels, BD, and Hall, SD. (2001) The effect of rifampin administration on the disposition of fexofenadine. *Clin Pharmacol Ther* **69**:114-121.

Ho RH and Kim RB (2005) Transporters and drug therapy: implications for drug disposition and disease. *Clin Pharmacol Ther* **78**:260-77.

Hofmann, U, Seiler, M, Drescher, S, and Fromm, MF. (2002) Determination of fexofenadine in human plasma and urine by liquid chromatography-mass spectrometry. *J Chromatogr B* **766**:227-237.

Imanaga J, Kotegawa T, Imai H, Tsutsumi K, Yoshizato T, Ohyama T, Shirasaka Y, Tamai I, Tateishi T and Ohashi K (2011) The effects of the SLCO2B1 c.1457C > T polymorphism and apple juice on the pharmacokinetics of fexofenadine and midazolam in humans. *Pharmacogenet Genomics* **21**:84-93.

Ishiguro N, Maeda K, Kishimoto W, Saito A, Harada A, Ebner T, Roth W, Igarashi T, and Sugiyama Y. (2006) Predominant contribution of OATP1B3 to the hepatic uptake of telmisartan, an angiotensin II receptor antagonist, in humans. *Drug Metab Dispos* **34**:1109-15.

Jajoo HK, Mayol RF, LaBudde JA, and Blair IA (1989) Metabolism of the antianxiety drug buspirone in human subjects. *Drug Metab Dispos* **17**:634-40.

Kamath AV, Yao M, Zhang Y and Chong S. (2005) Effect of fruit juices on the oral bioavailability of fexofenadine in rats. *J Pharm Sci* **94**:233-239.

Kaukonen KM, Olkkola KT, and Neuvonen PJ (1999) Fluconazole but not itraconazole decreases the metabolism of losartan to E-3174. *Eur J Clin Pharmacol* **53**:445-9.

Kazierad DJ, Martin DE, Blum RA, Tenero DM, Ilson B, Boike SC, Etheredge R, and Jorkasky DK (1997) Effect of fluconazole on the pharmacokinetics of eprosartan and losartan in healthy male volunteers. *Clin Pharmacol Ther* **62**:417-25.

Kharasch ED, Walker A, Hoffer C, and Sheffels P (2005) Evaluation of first-pass cytochrome P4503A (CYP3A) and P-glycoprotein activities using alfentanil and fexofenadine in combination. *J Clin Pharmacol* **45**:79-88.

Kim, RB (2003) Organic anion-transporting polypeptide (OATP) transporter family and drug disposition. *Eur J Clin Investigation* **33**:1-5.

Kivistö KT, Lamberg TS, Kantola T, and Neuvonen PJ (1997) Plasma buspirone concentrations are greatly increased by erythromycin and itraconazole. *Clin Pharmacol Ther* **62**:348-54.

König J, Cui Y, Nies AT, and Keppler D (2000a) Localization and genomic organization of a new hepatocellular organic anion transporting polypeptide. *J Biol Chem*. **275**:23161-8.

König J, Cui Y, Nies AT, and Keppler D (2000b) A novel human organic anion transporting polypeptide localized to the basolateral hepatocyte membrane. *Am J Physiol Gastrointest Liver Physiol* **278**:G156-G164.

Kullak-Ublick GA, Ismair MG, Stieger B, Landmann L, Huber R, Pizzagalli F, Fattinger K, Meier PJ and Hagenbuch B (2001) Organic anion-transporting polypeptide B (OATP-B) and its functional comparison with three other OATPs of human liver. *Gastroenterology* **120**:525-533.

Laine K, Ahokoski O, Huupponen R, Hänninen J, Palovaara S, Ruuskanen J, Björklund H, Anttila M, and Rouru J (2003) Effect of the novel anxiolytic drug deramciclane on the pharmacokinetics and pharmacodynamics of the CYP3A4 probe drug buspirone. *Eur J Clin Pharmacol* **59**:761-6.

Lamberg TS, Kivistö KT, and Neuvonen PJ (1998) Concentrations and effects of buspirone are considerably reduced by rifampicin. *Br J Clin Pharmacol* **45**:381-5.

Lee CR, Pieper JA, Hinderliter AL, Blaisdell JA, and Goldstein JA (2003) Losartan and E3174 pharmacokinetics in cytochrome P450 2C9*1/*1, *1/*2, and *1/*3 individuals. *Pharmacotherapy* **23**:720-5.

Lee W, Glaeser H, Smith LH, Roberts RL, Moeckel GW, Gervasini G, Leake BF, and Kim RB (2005) Polymorphisms in human organic anion-transporting polypeptide 1A2 (OATP1A2): implications for altered drug disposition and central nervous system drug entry. *J Biol Chem* **280**:9610-9617.

Lin JH and Yamazaki M. (2003) Role of P-glycoprotein in pharmacokinetics. *Clin Pharmacokinet* **42**: 59-98.

Lippert C, Ling J, Brown P, Burmaster S, Eller M and Cheng L (1995) Mass balance and pharmacokinetics of MDL 16455A in healthy male volunteers [abstract]. *Pharm Res* **12**:S390.

Lown KS, Mayo RR, Leichtman AB, Hsiao HL, Turgeon DK, Schmiedlin-Ren P, Brown MB, Guo W, Rossi SJ, Benet LZ, and Watkins PB (1997) Role of intestinal P-glycoprotein (mdr1) in interpatient variation in the oral bioavailability of cyclosporine. *Clin Pharmacol Ther* **62**:248-60.

Matsushima S, Maeda K, Ishiguro N, Igarashi T. and Sugiyama Y. (2008) Investigation of the inhibitory effects of various drugs on the hepatic uptake of fexofenadine in humans. *Drug Met Dispos* **36**: 663-669.

Meadowcroft AM, Williamson KM, Patterson JH, Hinderliter AL, and Pieper JA (1999) The effects of fluvastatin, a CYP2C9 inhibitor, on losartan pharmacokinetics in healthy volunteers. *J Clin Pharmacol* **39**:418-24.

Miners JO and Birkett DJ (1998) Cytochrome P4502C9: an enzyme of major importance in human drug metabolism. *Br J Clin Pharmacol* **45**:525-38.

Moghadamnia AA, Rostami-Hodjegan A, Abdul-Manap R, Wright CE, Morice AH, and Tucker GT (2003) Physiologically based modeling of inhibition of metabolism and assessment of the relative potency of drug and metabolite: dextromethorphan vs. dextrorphan using quinidine inhibition. *Br J Clin Pharmacol* **56**:57-67.

Müller F, Fromm MF (2011) Transporter-mediated drug-drug interactions. *Pharmacogenomics* **12**:1017-37.

Nakagomi-Hagihara R, Nakai D, Kawai K, Yoshigae Y, Tokui T, Abe T, and Ikeda T (2006) OATP1B1, OATP1B3, and mrp2 are involved in hepatobiliary transport of olmesartan, a novel angiotensin II blocker. *Drug Metab Dispos* **34**:862-9.

Niemi M, Backman JT, Kajosaari LI, Leathart JB, Neuvonen M, Daly AK, Eichelbaum M, Kivistö KT, and Neuvonen PJ (2005) Polymorphic organic anion transporting polypeptide 1B1 is a major determinant of repaglinide pharmacokinetics. *Clin Pharmacol Ther* **77**:468-478.

Nozawa T, Imai K, Nezu J, Tsuji A, and Tamai I (2004) Functional characterization of pH-sensitive organic anion transporting polypeptide OATP-B in humans. *J Pharmacol Exp Ther* **308**:438-45.

Pacyniak E, Roth M, Hagenbuch B and Guo GL (2010) Mechanism of polybrominated diphenyl ether uptake into the liver: PBDE congeners are substrates of human hepatic OATP transporters. *Toxicol Sci* **115**:344-353.

Parasrampuria DA, Lantz MV and Benet LZ (2001) A human lymphocyte based ex vivo assay to study the effect of drugs on P-glycoprotein (P-gp) function. *Pharm Res* **18**:39-44.

Peng WX, Li HD, and Zhou HH (2004) Effect of daidzein on CYP1A2 activity and pharmacokinetics of theophylline in healthy volunteers. *Eur J Clin Pharmacol* **59**:237-41.

Petsalo A, Turpeinen M, Pelkonen O, and Tolonen A. (2008) Analysis of nine drugs and their cytochrome P450-specific probe metabolites from urine by liquid chromatography-tandem mass spectrometry utilizing sub 2 microm particle size column. *J Chromatogr A* **1215**:107-15.

Qiang F, Lee BJ, Lee W and Han HK (2009) Pharmacokinetic drug interaction between fexofenadine and fluvastatin mediated by organic anion-transporting polypeptides in rats. *Eur J Pharm Sci* **37**:413-417.

Reed, GA, Flynn, CA, and Hagenbuch, B. (2010) Fexofenadine is not a substrate for OATP2B1. *Basic and Clin. Pharm.& Toxicol* **107**(Supplement 1): 541.

Rendic, S and Di Carlo, FJ. (1997) Human cytochrome P450 enzymes: a status report summarizing their reactions, substrates, inducers, and inhibitors. *Drug Metab Rev* **29**:413-580.

Robertson SM, Davey RT, Voell J, Formentini E, Alfaro RM, and Penzak SR (2008) Effect of Ginkgo biloba extract on lopinavir, midazolam and fexofenadine pharmacokinetics in healthy subjects. *Curr Med Res Opin* **24**:591-9.

Roth, M, Timmerman, BN and Hagenbuch B. (2011) Interactions of green tea catechins with organic anion-transporting polypeptides. *Drug Metab Dispos* **39**:920-6.

Russell T, Stoltz M, and Weir S (1998) Pharmacokinetics, pharmacodynamics, and tolerance of single- and multiple-dose fexofenadine hydrochloride in healthy male volunteers. *Clin Pharmacol Ther* **64**:612-21.

Ryu JY, Song IS, Sunwoo YE, Shon JH, Liu KH, Cha IJ and Shin JG (2007) Development of the "Inje cocktail" for high-throughput evaluation of five human cytochrome P450 isoforms in vivo. *Clin Pharmacol Ther* **82**:531-540.

Scott RJ, Palmer J, Lewis IA, and Pleasance S (1999) Determination of a 'GW cocktail' of cytochrome P450 probe substrates and their metabolites in plasma and urine using automated solid phase extraction and fast gradient liquid

chromatography tandem mass spectrometry. *Rapid Commun Mass Spectrom* **13**:2305-19.

Shimizu M, Fuse K, Okudaria K, Nishigaki R, Maeda K, Kusuhara H and Sugiyama Y (2005) Contribution of OATP (organic anion-transporting polypeptide) family transporters to the hepatic uptake of fexofenadine in humans. *Drug Metab Dispos* **33**: 1477-1481.

Shimizu M, Uno T, Sugawara K and Tateishi T (2006) Effects of itraconazole and diltiazem on the pharmacokinetics of fexofenadine, a substrate of P-glycoprotein. *Br J Clin Pharmacol* **61**:538-544.

St-Pierre MV, Ugele B, Hagenbuch B, Meier PJ, and Stallmach T (2002) Characterization of an organic anion transporting polypeptide (OATP-B) in human placenta. *J Clin Endocr Metab* **87**:1856-1863.

Stearns RA, Chakravarty PK, Chen R, and Chiu SH. (1995) Biotransformation of losartan to its active carboxylic acid metabolite in human liver microsomes: Role of cytochrome P4502C and 3A subfamily members. *Drug Metab Dispos* **23**:207-15.

Stoltz, M, Arumugham, T, Lippert, C, Yu, D, Bhargava, V, Eller, M, and Weir, S (1997) Effect of food on the the bioavailability of fexofenadine hydrochloride (MDL 16455A). *Biopharma & Drug Dispos* 18: 645-8.

Streetman DS, Bleakley JF, Kim JS, Nafziger AN, Leeder JS, Gaedigk A, Gotschall R, Kearns GL, and Bertino JS Jr (2000) Combined phenotypic assessment of CYP1A2, CYP2C19, CYP2D6, CYP3A, N-acetyltransferase-2, and xanthine oxidase with the "Cooperstown cocktail". *Clin Pharmacol Ther* **68**:375-83.

Strohmenger HU, Lindner KH, Wienen W, and Radermacher P (1998) Effects of an angiotensin II antagonist on organ perfusion during the post-resuscitation phase in pigs. *Crit Care* **2**:49-55.

Su Y, Zhang X, and Sinko PJ (2004) Human organic anion-transporting polypeptide OATP-A (SLC21A3) acts in concert with P-glycoprotein and multidrug resistance protein 2 in the vectorial transport of Saquinavir in Hep G2 cells. *Mol Pharm* **1**:49-56.

Tamai I, Nezu J, Ichino H, Sai Y, Oku A, Shimane M, and Tsuji A. (2000) Molecular identification and characterization of novel members of the human organic anion transporter (OATP) family. *Biochem Biophys Res Comm* **273**:251-260.

Taimi I, Nowzawa T, Koshida M, Nezu J, Sai Y, and Tsuji A (2001) Functional characterization of human organic anion transporting polypeptide B (OATP-B) in comparison with liver specific OATP-C. *Pharm Res* **18**:1262-1269.

Timmermans PB, Wong PC, Chiu AT, Herblin WF, Benfield P, Carini DJ, Lee RJ, Wexler RR, Saye JA, and Smith RD (1993) Angiotensin II receptors and angiotensin II receptor antagonists. *Pharmacol Rev* **45**:205-51.

Transon C, Leemann T, Vogt N, and Dayer P (1995) In vivo inhibition profile of cytochrome P450TB (CYP2C9) by (+/-)-fluvastatin. *Clin Pharmacol Ther* **58**:412-7.

Uno, T, Yasui-Furukori, N, Takahata, T, Sugawara, K, and Tateishi, T. (2004) Liquid chromatographic determination of fexofenadine in human plasma with fluorescence detection. *J Pharmaceut Biomed* **35**:937-947.

van Heeswijk RP, Bourbeau M, Campbell P, Seguin I, Chauhan BM, Foster BC, and Cameron DW (2006) Time-dependent interaction between lopinavir/ritonavir and fexofenadine. *J Clin Pharmacol* **46**:758-67.

Wang B, Wang J, Huang SQ, Su HH, and Zhou SF (2009) Genetic polymorphism of the human cytochrome P450 2C9 gene and its clinical significance. *Curr Drug Metab* **10**:781-834.

Wang Z, Hamman MA, Huang SM, Lesko LJ and Hall SD (2002) Effect of St John's wort on the pharmacokinetics of fexofenadine. *Clin Pharmacol Ther* **71**:414-420.

Yamane, N, Tozuka, Z, Sugiyama, Y, Tanimoto, T, Yamazaki, A, and Kumagai, Y. (2007) Microdose clinical trial: Quantitation determination of fexofenadine in human plasma using liquid chromatography/electrospray ionization tandem mass spectrometry. *J Chromatogr B* **858**:118-128.

Yamada A, Maeda K, Kamiyama E, Sugiyama D, Kondo T, Shiroyanagi Y, Nakazawa H, Okano T, Adachi M, Schuetz JD, Adachi Y, Hu Z, Kusuhara H, and Sugiyama Y (2007) Multiple human isoforms of drug transporters contribute to the hepatic and renal transport of olmesartan, a selective antagonist of the angiotensin II AT1-receptor. *Drug Metab Dispos* **35**:2166-76.

Yamashiro W, Maeda K, Hirouchi M, Adachi Y, Hu Z, and Sugiyama Y (2006) Involvement of transporters in the hepatic uptake and biliary excretion of valsartan, a

selective antagonist of the angiotensin II AT1-receptor, in humans. *Drug Metab Dispo* **34**:1247-54.

Yasar U, Dahl ML, Christensen M, and Eliasson E (2002) Intra-individual variability in urinary losartan oxidation ratio, an in vivo marker of CYP2C9 activity. *Br J Clin Pharmacol* **54**:183-5.

Yasui-Furukori N, Uno T, Sugawara K and Tateishi T (2005) Different effects of three transporting inhibitors, verapamil, cimetidine, and probenecid, on fexofenadine pharmacokinetics. *Clin Pharmacol Ther* **77**:17-23.

Yocca, FD (1990) Neurochemistry and neurophysiology of buspirone and gepirone: interactions at presynaptic and postsynaptic 5-HT1A receptors. *J Clin Psychopharmacol* **10**(3 Suppl):6S-12S.

Yun CH, Lee HS, Lee H, Rho JK, Jeong HG, and Guengerich FP (1995) Oxidation of the angiotensin II receptor antagonist losartan (DuP 753) in human liver microsomes. Role of cytochrome P4503A(4) in formation of the active metabolite EXP3174. *Drug Metab Dispos* **23**:285-9.

Zhang L, Huang SM, and Lesko LJ (2011) Transporter-mediated drug-drug interactions. *Clin Pharmacol Ther* 89: 481-4.

Zhu B, Ou-Yang DS, Chen XP, Huang SL, Tan ZR, He N, and Zhou HH (2001) Assessment of cytochrome P450 activity by a five-drug cocktail approach. *Clin Pharmacol Ther* **70**:455-61.

Appendices:

Appendix I: Citations of Publication

Flynn, CA, Alnouti, Y and Reed, GA (2011) Quantification of the transporter substrate fexofenadine in cell lysates by liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom* **25**:2361-2366.

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