The Hepatobiliary Transport of Rosuvastatin *In Vivo*

EBBA BERGMAN
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Abstract

In vivo studies of hepatobiliary disposition are challenging. The hepatobiliary system is complex, as its physiological localization, complex cellular structure with numerous transporters and enzymes, and the interindividual variability in protein expression and biliary flow will all affect the in vivo disposition of a drug under investigation. The research included in this thesis has focused on the involvement of hepatic transport proteins in the hepatobiliary disposition of rosuvastatin. The impact that several transport inhibitors had on the pharmacokinetics of rosuvastatin was investigated in healthy volunteers and in pigs. The effects were considerable, following inhibition of sinusoidal transport proteins by cyclosporine and rifampicin. These inhibitors significantly reduced the hepatic extraction of rosuvastatin by 50 and 35%, respectively, and the plasma exposure increased by factors of 9.1 and 6.3, respectively. Drug-drug interactions (DDI) resulting in markedly higher plasma exposures are important from a drug safety perspective as increased extrahepatic exposure of statins is associated with an increased risk of severe side-effects, such as myopathy which in rare cases could develop into rhabdomyolysis. The DDI caused by cyclosporine and rifampicin can probably be attributed to inhibition of hepatic uptake transporters. In contrast, inhibition of canalicular transporters by imatinib did not significantly affect the pharmacokinetics of rosuvastatin, which suggests that the intracellular concentration of the inhibitor in the hepatocyte was insufficient to affect the transport of rosuvastatin, or that imatinib is not a sufficiently potent inhibitor in vivo. Furthermore, gemfibrozil administered as a single dose into the jejunum in healthy volunteers and pigs did not affect the plasma or biliary pharmacokinetics of rosuvastatin. The previously reported DDI in humans upon repeated dosing with gemfibrozil might be explained by the accumulation of metabolites able to affect the disposition of rosuvastatin. The investigations presented in this thesis conclude that transport proteins are of considerable importance for the hepatobiliary disposition of rosuvastatin in vivo. The Loc-I-Gut catheter can be applied for the investigation of biliary accumulation and to determine bile specific metabolites, however it has limitations when conducting quantitative measurements. In the porcine model, hepatic bile can be collected for up to six hours and enables the determination of the hepatic extraction in vivo.

Keywords: Rosuvastatin, Biliary excretion, Transport inhibition, Pharmacokinetics, Drug–drug interactions, Hepatobiliary transport, Organic anion transporting polypeptide, OATP, Statins, Gemfibrozil, Cyclosporine, Imatinib, Rifampicin, Canalicular transport, Sinusoidal transport, Hepatic uptake, Hepatic extraction

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Till David och Calle
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals assigned below.


IV Bergman, E., Hedeland, M., Bondesson, U. and Lennernäs, H. The Effect of Acute Administration of Rifampicin and Imatinib on the Enterohepatic Transport of Rosuvastatin In Vivo. In manuscript.

Reprints were made with permission from the respective publishers.
Additional papers not included in this thesis:


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# Abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ASBT</td>
<td>Apical sodium-dependent bile acid transporter</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BCRP</td>
<td>Breast cancer resistance protein</td>
</tr>
<tr>
<td>BMDP</td>
<td>Brain multidrug resistance protein</td>
</tr>
<tr>
<td>BSEP</td>
<td>Bile salt export pump</td>
</tr>
<tr>
<td>CA</td>
<td>Cholic acid</td>
</tr>
<tr>
<td>Caco-2</td>
<td>Human colon adenocarcinoma cell</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>CDCA</td>
<td>Chenodeoxycholic acid</td>
</tr>
<tr>
<td>CL</td>
<td>Clearance</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximum concentration</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DCA</td>
<td>Deoxycholic acid</td>
</tr>
<tr>
<td>DDI</td>
<td>Drug-drug interaction</td>
</tr>
<tr>
<td>E&lt;sub&gt;G&lt;/sub&gt;</td>
<td>Gut extraction</td>
</tr>
<tr>
<td>E&lt;sub&gt;H&lt;/sub&gt;</td>
<td>Hepatic extraction</td>
</tr>
<tr>
<td>F</td>
<td>Bioavailability</td>
</tr>
<tr>
<td>f&lt;sub&gt;a&lt;/sub&gt;</td>
<td>Fraction absorbed</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and drug administration</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>HLM</td>
<td>Human liver microsomes</td>
</tr>
<tr>
<td>HMG-Co A</td>
<td>3-hydroxy-3-methylglutaryl coenzyme A</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Inhibitor concentration 50</td>
</tr>
<tr>
<td>IJ</td>
<td>Intrajejunal</td>
</tr>
<tr>
<td>LLC-PK1</td>
<td>Pig kidney epithelial cell line</td>
</tr>
<tr>
<td>I.V.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>J&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximum transport velocity</td>
</tr>
<tr>
<td>K&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Inhibitor constant</td>
</tr>
<tr>
<td>K&lt;sub&gt;m&lt;/sub&gt;</td>
<td>Michaelis-Menten constant</td>
</tr>
<tr>
<td>LCA</td>
<td>Lithocholic acid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>MATE</td>
<td>Multidrug and toxin extrusion protein</td>
</tr>
<tr>
<td>MDCKII</td>
<td>Madin-Darby canine kidney strain II</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug resistance protein</td>
</tr>
<tr>
<td>MMC</td>
<td>Migratory motor complex</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MRP</td>
<td>Multidrug-resistance associated protein</td>
</tr>
<tr>
<td>NTCP</td>
<td>Sodium-taurocholate cotransporting polypeptide</td>
</tr>
<tr>
<td>OAT</td>
<td>Organic anion transporter</td>
</tr>
<tr>
<td>OATP</td>
<td>Organic anion transporting polypeptide</td>
</tr>
<tr>
<td>OCT</td>
<td>Organic cation transporter</td>
</tr>
<tr>
<td>OST</td>
<td>Organic solute transporter</td>
</tr>
<tr>
<td>PBPK</td>
<td>Physiologically based pharmacokinetic model</td>
</tr>
<tr>
<td>PEPT</td>
<td>Peptide transporter</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SLC</td>
<td>Solute-carrier</td>
</tr>
<tr>
<td>SNP</td>
<td>Single-nucleotide polymorphism</td>
</tr>
<tr>
<td>UGP</td>
<td>UDP glucuronosyltransferase</td>
</tr>
<tr>
<td>VF</td>
<td>Femoral vein</td>
</tr>
<tr>
<td>VH</td>
<td>Hepatic vein</td>
</tr>
<tr>
<td>VP</td>
<td>Portal vein</td>
</tr>
<tr>
<td>Qhl</td>
<td>Hepatic blood flow</td>
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</tbody>
</table>
1. Introduction

The human body has evolved to protect us from harmful compounds and to ensure that nutrients are absorbed. One challenge in drug development is to utilize and overcome these physiological barriers and direct an exogenous compound to the site of its pharmacological activity. Oral administration is the most common way to administer drugs as it is associated with low production costs and is convenient for the patient. However, before the drug arrives at the desired site, numerous steps have to be overcome before it reaches the systemic circulation following ingestion. The term used to describe the fraction of an orally administered dose that reaches the target site is bioavailability (F). Bioavailability is defined as the fraction of the administered drug that reaches the systemic circulation; it can be calculated using Equation 1 (Figure 1).

Before a drug becomes available for absorption, the solid tablet has to disintegrate into smaller particles, these are subsequently dissolved in the gastrointestinal fluid, releasing the drug molecules. The formulation of the tablet determines the disintegration rate and physiological properties such as gastric empting, the gastrointestinal transit time and the pH of the gastrointestinal fluid. The rate at which dissolution from solid particles to drug molecules occurs will depend on the physiochemical properties of the drug and particles, the formulation and the environment of the gastrointestinal tract (for example the pH and bile acid content). A drug molecule in solution might be subjected to enzymatic degradation and/or precipitation. Metabolism and transport efflux can limit the fraction of the drug that is absorbed (F_{a}) and transported over the gastrointestinal epithelium (1-E_{C}) and into the portal vein (Figure 1). Subsequently, the liver will extract a fraction of the molecules (E_{H}) in the perfusing blood by metabolism and/or biliary excretion, however, the remaining molecules which have escaped all the steps of Figure 1, become available to the systemic circulation (Figure 1). Bile is produced by the liver to aid in the absorption of fat from the gastrointestinal tract, but it also functions as an elimination pathway for endo- and exogenous compounds. Bile is emptied into the distal duodenum, where the bile acids emulsify the ingested fat. The bile acids are re-absorbed and transported back to the liver; this shunt is called the enterohepatic circulation, and for some drugs this can be an important part of drug disposition (Figure 1).

Alterations of the processes depicted in (Figure 1) might influence the bioavailability of an administered drug, depending on which of the processes is the rate-liming step.
Figure 1. The challenges facing a drug molecule from the time it is ingested to the time it reaches the systemic circulation.

\[ F = f_a (1 - E_G) (1 - E_H) \]  

The disposition patterns of drug molecules vary, however, passage through a biological membrane is a common feature. These membranes are formed by tightly fitted cells, and separated by tight junctions (Figure 2). Cell membranes are predominantly composed of a double layer of amphipathic phospholipids, which form a lipophilic shield, preferably enabling lipophilic drug molecules to diffuse across it in a passive manner (transcellular transport) (Figure 2). The extent of passive diffusion will be dependent on the physiochemical properties of the drug molecule, such as the lipophilicity and the degree of ionization. Hydrophilic compounds, however, can pass a physiological membrane by binding to a transport protein located on the cell surface (Figure 2). Other means of passing a biological membrane are paracellular transport and endocytosis (Figure 2). Transport via transport proteins can either be passive (meaning no energy is required) or active (energy is required). Active transport is further divided into primary or secondary transport, depending on whether the transport protein itself generates the energy required for the transport (primary transport) or the energy is provided by a secondary component (secondary transport) (Figure 2). Efflux transport proteins direct their substrates in the opposite direction to influx ones, and thus counteract the absorption process across a biological membrane. The number of transport proteins at the cell membrane is limited, making transport by this means a saturable process with a maximum transport capacity.
The pharmacological target of rosuvastatin is located at the endoplasmatic reticulum of the liver cell (hepatocyte), therefore a high hepatic extraction is preferable. Furthermore, an elevated extrahepatic exposure is associated with severe side-effects, such as myopathy and rhabdomyolysis. The hepatic extraction of rosuvastatin ($E_H = 0.63$ humans) is mediated by multiple hepatic uptake transporters, and alterations to their transport capacities affect the pharmacokinetics and increase the risk of severe side-effects. It is, therefore, important to investigate these transport processes in vivo and to study their susceptibility to alteration following co-administration with inhibitors of the transporters involved.

1.1. The hepatobiliary system

The liver is the largest visceral organ in the body. It is involved in the elimination of endogenous and exogenous compounds. The liver is also responsible for the storage of nutrients, the production of bile acids and the excretion of bile. The gall bladder is situated in conjunction with the liver and the bile ducts are dispersed throughout the liver in a tree-like network (Figure 3A). The hepatocytes are arranged in subunits called the sinusoids. The subcellular arrangement of the sinusoids enables a high exchange of molecules between the perfusing blood ($Q_H \sim 1350$ ml/min) and the hepatocytes (Figure 3B). The hepatocytes are polarized cells, i.e. the cells have a sinusoidal/basolateral membrane facing the blood and a canalicular membrane facing the bile canaliculus (Figure 4). These two membranes of the hepatocytes differ in their lipid composition and are separated by tight junctions (Figure 4). The flow of blood and bile in the sinusoid is divergent (Figure 3B). The bile produced by the hepatocyte empies into the bile canaliculus and is transported to the bile duct, which is formed of cholangiocytes (Figure 3B). The bile ducts further combine to form large ducts and, finally, merge into the common hepatic duct (Figure 3A).
The transport proteins of the hepatocyte are presented in (Figure 4) and are either members of the solute-carrier (SLC) or the ATP-binding cassette (ABC) transporters superfamily. Transporters of the SLC family transport its substrates by facilitated diffusion or secondary transport mechanisms (symporter and antiporter). The human genome is considered to consist of at least 362 functional coding SLC genes and is divided into 55 subfamilies. Transport by the ABC family is driven by primary transport, mediated by ATP hydrolysis. Humans have 48 human ABC transporters which are divided into seven ABC subfamilies. The nomenclature of transporter proteins have changed over the years and examples of past names of the transporters commonly mentioned in the thesis are presented in Table 1.

![Diagram of human hepatobiliary system]

**Figure 3.** The hepatobiliary system in humans (A) and the cellular arrangement of the liver, called the sinusoids (B). The arrows in Figure 3B indicate the direction of the blood perfusing the liver and the direction of bile from the bile canaliculi to the bile duct.
Figure 4. Various transport proteins, which are able to transport their substrates in and out of the cell, are expressed on the sinusoidal and canalicular membrane of the hepatocytes.

Table 1. Examples of past nomenclature of the drug transporters commonly mentioned in the thesis along with their present name.

<table>
<thead>
<tr>
<th>Present nomenclature</th>
<th>Past nomenclature</th>
</tr>
</thead>
<tbody>
<tr>
<td>OATP1B1</td>
<td>LST-1, OAT-P-C, OATP2, OATP6</td>
</tr>
<tr>
<td>OATP1B3</td>
<td>LST-2, OATP8</td>
</tr>
<tr>
<td>OATP1A2</td>
<td>OATP-A, OATP-1, OATP</td>
</tr>
<tr>
<td>OATP2B1</td>
<td>OATP-B</td>
</tr>
<tr>
<td>NTCP</td>
<td>NTCP1, LBAT</td>
</tr>
<tr>
<td>ASBET</td>
<td>NTCP2, IBAT, ISBT</td>
</tr>
<tr>
<td>ABCB1, MDR1</td>
<td>P-glycoprotein, PGY1, GP170</td>
</tr>
<tr>
<td>ABCB11, MDR3, NTCP</td>
<td>Sister P-glycoprotein</td>
</tr>
<tr>
<td>ABCC2, MRP2</td>
<td>cMAOT</td>
</tr>
<tr>
<td>ABCC3, MRP3</td>
<td>cMAOT2, MOAT-D</td>
</tr>
<tr>
<td>ABCG2, BCRP</td>
<td>MXR, ABCP</td>
</tr>
</tbody>
</table>
1.1.1. Transport proteins located on the sinusoidal membrane of the hepatocyte

The transport proteins of the sinusoidal membrane of the hepatocyte are presented in (Figure 4). Both influx and efflux transporters are expressed on the sinusoidal membrane of the hepatocyte.

Organic anion transporting polypeptide (OATP)

Of the eleven identified proteins of the human OATP-family, four are considered to be expressed at the basolateral membrane of the hepatocyte, namely OATP1B1, OATP1B3, OATP2B1 and OATP1A2. In addition to being expressed in the liver, OATP transporters are expressed in various tissues throughout the body, such as the small intestine, blood brain barrier (BBB), placenta and kidney, and thus play an important role in the disposition of its substrates in the body. This transport family is known for its broad substrate specificity of organic amphipathic compounds, such as taurocholate, thyroxine (T4) hormone and fexofenadine. Predictions of the structure of OATPs indicate that they are composed of 9 to 12 transmembrane domains of 643 to 722 amino acids. Other characteristics of OATPs are a large fifth extracellular loop and several putative glycosylation sites.

OATP1B1 (SLCO1B1) and OATP1B3 (SLCO1B3) are believed to be specifically expressed in the liver and are localized on the basolateral membrane of the hepatocytes; they are not present in the cholangiocytes. OATP1B3 share an 80% homology in the amino acid sequence with OATP1B1 and have overlapping substrate specificity, although specific differences exist, such as digoxin and paclitaxel, which have been shown to be exclusively transported by OATP1B3. Immunoassay analysis of the protein expression of OATP1B3 has revealed that the expression varies throughout the liver. The hepatocytes lining the central vein have a higher expression of the transporter compared with the hepatocytes lining the portal vein.

In 1995, OATP1A2 (SLCO1A2) became the first human OATP-transporter identified and high mRNA levels were detected in the brain but also in the liver and the kidney. Hepatic OATP1A2 was shown by Lee et al to be exclusively expressed in the cholangiocytes. The presence of OATP1A2 in the duodenum suggests that it might be involved in the intestinal absorption of its substrates. OATP2B1 (SLCO2B1) mRNA was highest in the liver, but was also expressed in various tissues throughout the body, such as the spleen, placenta and pancreas. OATP2B1 is considered to have a narrower substrate specificity than the previously mentioned members of the OATP family, for instance, taurocholate and glycocholate are not substrates of OATP2B1. In the hepatocyte OATP2B1 is exclusively expressed on the basolateral membrane.
Sodium-taurocholate cotransporting polypeptide (NTCP)
NTCP (SLC10A1) plays an important part of in the enterohepatic circulation of bile acids and is considered to be the transporter responsible for the sodium dependent hepatic uptake of bile acids such as taurocholate. Bile acids are the primary substrates of this 362 amino acid long protein, however NTCP-mediated transport of estrone-3-sulfate and rosuvastatin have been reported.

Multidrug-resistance associated protein (MRP)
The distribution pattern of the MRP family in the body imply that they are of importance in cellular defense mechanisms. At the basolateral membrane of the hepatocyte, MRP1 and MRP3 to MRP6 are expressed. The substrates of the MRP family display a wide range of chemical structures including endogenous compounds such as bile acids, prostaglandins, methotrexate and folate. MRP3 (ABCC3) and MRP4 (ABCC4) have been suggested to be important transporters in bile acid homeostasis and are up-regulated during cholestasis in mice and humans, respectively.

Organic solute transporter (OST)
In 2001, the OSTα and OSTβ were cloned in the little skate, providing the missing piece in the puzzle of how bile acids absorbed by the enterocytes were transported over the basolateral membrane and into the mesenteric blood. In addition to the ileal enterocyte, the OSTα/β transporter is expressed in the hepatocyte, cholangiocyte and kidney proximal tubuli. OSTα and OSTβ together form a heterodimer and thus both subunits are necessary for substrate transport to occur. Besides bile acids, prostaglandin E₂, estrone-3-sulfate and digoxin have been identified as substrates of OSTα/β.

Organic cation transporter 1 (OCT1)
OCT1 (SLC22A1) is predominantly expressed in the human liver. This family of transporters exhibits a broad substrate specificity; prostaglandin E₂ and F₂α and metformin are examples of endogenous and exogenous substrates, respectively.

Organic anion transporter (OAT)
OAT2 (SLC22A7) is expressed at the basolateral membrane of the hepatocyte. As indicated by the name, organic anions are predominantly transported by OATs, however it is now recognized that even some organic cations are OAT substrates. Prostaglandin E₂ and estrone-3-sulfate are examples of substrates of OAT2.
Peptide transporter 1 (PEPT1)

PEPT1 (SLC15A1) is mainly expressed at the apical membrane of the enterocytes of the small intestine. However, PEPT1 mRNA has been detected in human liver tissue. Substrates of PEPT1 are dietary di- and tri-peptides, however several drugs with a peptidomimetic structure are also substrates of PEPT1, examples being β-lactam antibiotics and viral agents.

1.1.2. Transport proteins located on the canalicular membrane of the hepatocyte

Bile salt export pump (BSEP)

Owing to its sequence homology with MDR1 (former Pgp) BSEP (ABCB11) was in the beginning called sister of P-glycoprotein (SPGP). BSEP is involved in the excretion of bile acids and mutations in the ABCB11 gene results in progressive familial intrahepatic cholestasis of type 2. Drug molecules such as pravastatin are substrates of BSEP in vitro.

Breast cancer resistance protein (BCRP)

In 1998, Doyle and co-workers identified a 663 amino acid and ATP-dependant BCRP (ABCG2) in human MCF-7 breast cancer cells. The protein caused resistance to multiple chemotherapeutic compounds and was named accordingly. The specific localization of this half transporter to the canalicular membrane was shown by Maliepaard and co-workers using a BCRP specific antibody. Various tissues express BCRP, with the highest protein expression being observed in the placenta, small intestine and colon, and the BBB. In the hepatocyte, BCRP excretes its substrates into bile canaliculi and in Bcrp knockout mice the biliary clearance of pitavastatin was reduced by 90% compared to the clearance in the wild-type.

Multidrug resistance-associated protein 2 (MRP2)

In contrast to other members of the MRP-family, MRP2 (ABCC2) is localized on the canalicular membrane of the hepatocyte. It mediates the biliary excretion of sulfated and glucuronidated bile acids. Many xenobiotic compounds are also substrates of this transporter. Dubin-Johnson syndrome arises from mutation of in ABCC2 gene causing an inability to biliary excrete bilirubin due to absence of functional MRP2; commonly manifested as mild jaundice.

Multidrug resistance protein (MDR)

MDR1 (ABCB1) was first discovered in 1976, in the beginning MDR1 was believed to function as a defense mechanism against chemotherapeutic compounds in various cancer cells. Today MDR1 is known to be expressed in various tissues throughout the body, such as the intestine, kidney and
Numerous substrates of MDR1 have been detected with a wide range of physiochemical properties. MDR1 reduces the intracellular accumulation of its substrates. It is expressed on the apical surface of the enterocytes and on the canalicular membrane of the hepatocytes. MDR3 (ABCB4) is less investigated than its relative MDR1 and mutations in the ABCB4 gene give rise to progressive familial intrahepatic cholestasis type 3 and is manifested as impaired biliary phospholipid excretion.

**Multidrug and toxin extrusion protein (MATE)**

Two members of the MATE subfamily have been reported in humans, of which one, MATE1 (SLC47A1), is expressed in the hepatic canalicular membrane. It is also expressed in the kidney and skeletal muscle in humans. MATE1 mediates the elimination of organic cations such as cimetidine, quinidine and tetraethylammonium.
There are various methods to study drug transport in vitro and one example are transfected Xenopus laevis oocytes. The Xenopus laevis oocytes are transfected with cRNA coding for the transporter of interest which will be subsequently expressed on the plasma membrane of the oocytes. The uptake clearance (CL_{int,T}) by the transfected oocytes are measured at various substrate concentrations and can be described using Michaelis-Menten kinetics (Equation 2). The transporter-mediated passage over a biological membrane is a saturable process as the number of transport proteins on the cell membrane is limited. Therefore, the rate of transport will be reduced at higher substrate concentrations reaching a maximum (J_{max}). The presence of an inhibitor can reduce the transport of a substrate. Reversal inhibition can either be competitive or noncompetitive. The concentrations of a drug in vivo are generally well below the K_{m}-value and therefore are Equation 3 valid for both competitive and noncompetitive. Inhibitors are seldom specific and might thus inhibit multiple CL_{int,T} processes of the substrate.  

\[
CL_{int,T} = \frac{J_{max}}{K_m + C_u} \quad \text{ (Equation 2)}
\]

\[
CL_{int,T+i} = \frac{J_{max}}{K_m \left(1 + \frac{I_u}{K_i} \right)} \quad \text{ (Equation 3)}
\]

The effect of inhibition on transport processes can be investigated by measuring the transport rate of a substrate at various concentrations of an inhibitor. The inhibitor concentration resulting in 50% reduction of transport activity is termed the IC_{50}-value. This value expresses the potency of the inhibitor, the lower the IC_{50}-value the more potent inhibitor. The IC_{50}-value of an inhibitor is dependent on the substrate and the concentration of substrate used in the assay.

Here follows some examples of studies using different in vitro methods for investigating drug transport; Madine-Darby Canine Kidney (MDCK) cells and the pig kidney epithelial cell line (LLC-PK1) are examples of cells which forms monolayers and can be transfected with one or more transport proteins. The disappearance/accumulation can for examples be investigated in membranes vesicles expressing a specific transport protein or transfected human embryonic kidney (HEK293). Human colon adenocarcinoma cells (Caco-2) and hepatocytes (isolated or double-sandwich cultured) are examples of non-transfectant systems with several transport proteins present.
1.1.3. The composition of bile

Bile is produced by the hepatocytes in the liver and excreted into the bile canaliculus. The dark color of bile derives from bilirubin which is an end product of hemoglobin destruction. The bile canaliculi are connected to the terminal bile ducts, which terminate in the common hepatic duct. The hepatic bile is subsequently stored in the gall bladder via the cystic duct or emptied into the duodenal ampulla via the common bile duct (Figure 3A). During its travel down the branches of the biliary duct tree, the lining cholangiocytes modulate the canalicular bile by secretion and re-absorption of electrolytes and water in response to hormones, peptides, the nervous system and biliary components. The cholangiocytes can reabsorb bile acids from the bile ducts. The reabsorption of bile acids by the cholangiocytes back into the blood is called the cholangiohepatic shunt. Transport proteins on the apical and basolateral membrane of the cholangiocytes mediate this shunt, though the more lipophilic bile acids also can be transported via passive diffusion. The composition of bile varies depending on its location in the biliary system, however the largest component of bile is always water. In the gall bladder, bile acids are concentrated hence the concentration of bile acids in the gall bladder bile is about six times higher than hepatic bile.

Hepatocytes convert cholesterol into primary bile acids and the regulation of this synthesis is complex and mediated by nuclear receptors such as the farnesoid X receptor. Cholic acid (CA) or chenodeoxycholic acid (CDCA) are the bile acids synthesized from cholesterol. These two bile acids can be conjugated with an amino acid (taurine or glycine), glucuronidated or sulfated into conjugated bile acids which are less hepatotoxic and have a higher solubility than unconjugated bile acids. The transport of conjugated bile acids into the bile canaliculus is mediated by BSEP or MRP2. Following excretion into the small intestine, the conjugated bile acids can be dehydroxylated into secondary bile acids or deconjugated bile acids. Lithocholic acid (LCA) and deoxycholic acid (DCA) are examples of secondary bile acids.

1.1.4. The biliary flow and enterohepatic circulation

In the fasted state, hepatic bile can either be stored in the gall bladder or empty into the duodenum and the directory of these two pathways are highly variable. In 1980, Shaffer et al showed that the average fraction of an intravenously administered 99mTc-HIDA that was stored in the gall bladder in healthy volunteers was 70% and ranged from 13 to 97%. Furthermore, in 2004, Ghibellini and co-workers showed that the biliary excretion of Tc-99m mebrofenin differed significantly in four healthy volunteers in the fasted
state. For instance, no storage of Tc-99m mebrofenin was observed in the
gall bladder in one subject, whereas in another, no duodenal emptying of Tc-
99m mebrofenin was observed during the 180 minutes investigated. The
directory of the hepatic bile is dependent on the hepatic secretory pressure,
the muscular tone of the gall bladder and the resistance of the sphincter of
Oddi. Even though, bile primarily acts as an emulsifier of ingested fat, it
also empties into the duodenum in the fasted state in Phase IV of the
migratory motor complex (MMC). The MMC regulates the peristaltic
movement in the small intestine during the fasted state. The MMC consists
of four consecutive phases and appears in cycles of 120 minutes (range 50-
200 minutes). Lanzini et al, however, report on bile emptying into the
duodenum in other phases than Phase IV of the MMC in humans.
Ellenbogen et al reported that, for 2 to 5 h, the average number of the times
the gall bladder emptied was less than one in 18 healthy subjects in the
fasted state. In the fed state, the emptying of bile is mediated by
cholecystokinin (CCK) and acetylcholine, which is a response to the volume
changes of the intestine and to components in the ingested food.

The human body is designed to efficiently recycle its pool of bile acids
(≈3 g) about 4-12 times per day, with approximately 95% being reabsorbed.
Following the emulsification of ingested fat, the bile acids are absorbed by
apical sodium-dependent basolateral transpoter (ASBT, SLC10A2) located on
the apical membrane of the entrocyte in the ilium. The OSTα/OSTβ enables
the basolateral transport of the bile acids into the portal blood which will
transport them back to the liver. Once at the liver, bile acids are taken up by
the hepatocyte via sodium-dependent (NTCP) and sodium independent
(OATPs) uptake transporters, as mentioned previously. In general, the
conjugated bile acids are transported via NTCP and the unconjugated ones
are transported via OATPs and, to some extent, by passive diffusion. Once
the bile acids have re-entered the bile canaliculi, a transport mediated by
BSEP and MRP2, another round of enterohepatic circulation has begun. The
enterohepatic circulation can be utilized by drugs and is shown by multiple
peaks in their plasma concentration-time profiles.

The transporters involved in the disposition of bile acids are regulated to
maintain bile acid homeostasis. To prevent accumulation of hepatotoxic bile
acid accumulation inside the hepatocyte during cholestasis the expression of
bile acid uptake transporters is reduced along with an increased expression
of bile acid efflux transporters. Ntcp and Oatp1b1 were down-regulated and
Bsep, Mrp3 and Mdr1a/b were up-regulated in cholic acid-induced
cholestasis in mice. Duodenal transporters involved in the uptake and
excretion of bile acids are also affected by cholestasis as it has been reported
that the expressions of ASBT and BCRP are reduced in humans suffering
from obstructive cholestasis.
1.1.5. Investigation of biliary excretion of drugs in humans

The investigation of biliary excretion *in vivo* is challenging. Owing to the position of the gall bladder, collection of duodenal bile is the only non-invasive method available in healthy volunteers (Figure 3A). Before laparoscopy surgery, studies were performed on patients recovering from hepatic surgery having a temporary bile shunt. However, the biliary flow, composition of bile and transport protein expressions are affected by cholestasis and might, therefore, not be representative for healthy volunteers. Several methods have been developed to study biliary excretion by aspiration of duodenal bile using different intubation catheters with and without intact enterohepatic circulation. Several studies include CCK or caerulein, to stimulate gall bladder contraction, to correct for the fraction of drug retained in the gall bladder. The response to CCK was immediate (within ~5 min), however it might be variable and associated with discomfort for the subjects. Inclusion of amino acids in the intestinal infusion might also stimulate gall bladder contraction.

Inclusion of a bile and/or gall bladder marker substance, such as indocyanine green, ²⁹Tm-HIDA or ²⁹Tm-mebrofinin reduces the variability of the biliary excretion significantly. However, the consequences of disrupting the MMC using CCK to describe normal biliary expression processes has been questioned. Other means of investigating biliary excretion in humans are fecal recovery, preferably using a radiolabelled compound.

1.1.6. Investigation of biliary excretion of drugs in animals

Bile excretion studies are generally performed in laboratory animals such as the rat and mouse, using techniques such as isolated perfused liver and bile duct cannulation. In situ or in vivo studies are important for pharmacokinetic assessments though species differences have to be considered, for example, rats have no gall bladder. Knockout mice (Mdr1a/-/la- and Mdr1b/-/lb-, Abcg2/-/-) enable the involvement of a specific transport protein for the biliary clearance of drugs to be investigated. Eisai hyperbilirubinemic rats are Mrp2-deficient, therefore they are used to investigate the importance of Mrp2 in drug disposition. Combination knockout mice with several ABC transporters knockedout have been developed because of the transporters overlapping substrate specificites.

The pig was chosen as the laboratory animal for assessing the first-pass metabolism and hepatic disposition of verapamil by our research group in 2006. The pig was chosen because it is an omnivore, like humans, and it was proposed by Karali in 1995 to be a suitable animal for absorption studies.
due to its resemblance to humans with regard to its gastrointestinal physiology in comparison to smaller animals. In addition, the relative size of the liver and the hepatic fraction of the cardiac output are similar in humans and pigs. In comparison to humans in conventional pig strains, although the content in the Göttingen minipig was 2- to 3-fold higher than in humans. Studies on the applicability of pigs as a model animal for metabolisms are inconclusive, and are, most likely, dependent on the investigated metabolizing enzyme. The pig has been suggested to be a suitable model animal for investigation of CYP3A-mediated metabolism. For example, pigs, like humans, exhibit a stereoselective CY3A-mediated metabolism of R/S-verapamil.

Several drug transporters have been reported in pigs. MDR1 is expressed in the small intestine of Yucatan minipigs and in liver tissue from a Cotswold pig. MRP1 and MRP2 mRNA have also been detected in pig liver using RT-PCR. Eisenblätter et al identified high mRNA expression of a BCRP homolog at the BBB, and in kidney and lung tissue from pig, and moderate expression in liver, colon and small intestine. Pig BCRP (initially named brain multidrug resistance protein, BMDP) shares an 86% amino acid identity with human BCRP. Low levels of OATP1B1 mRNA were reported in liver tissue from one Cotswold pig, however OATP1A2 mRNA was present at higher levels. The amino acid sequence of porcine OATP1B1 and OATP1A2 shared an 85 and 73% identity with their respective human protein in a partial isolated segment.

1.1.7. Investigation of biliary excretion of drugs in vitro

The most advanced in vitro method with which to study the biliary clearance of drugs are sandwich-cultured hepatocytes. In this model bile ducts are formed when human or rat hepatocytes are cultured between two layers of for example collagen. Several transport proteins are expressed in the sandwich-cultured hepatocytes model at the respective physiological membranes i.e. OATPs are expressed on the sinusoidal membrane and MDR1 and MRP2 are localized to the canalicular membrane. Investigation of biliary clearance in vitro can also be performed with multi-transfected MDCKII cells.

1.1.8. Drug-drug interactions involving inhibition of drug transport

Upon multiple drug dosing regime a drug A may affect drug B, i.e. cause a drug-drug interaction (DDI). Reasons for DDI are many and might affect the pharmacokinetics and/or pharmacodynamics of drug B. DDI is not always a disadvantage and could potentially be utilized to maximize the therapeutic effect of drug B. The extent of a pharmacokinetic DDI can be expressed as...
the ratio of the plasma AUC with and without drug A (Equation 4). What is classified as a clinically relevant DDI is dependent on the therapeutic range, however, a general R-value of above 2.5-5 is likely to be of clinical relevance.\textsuperscript{128, 129} For the narrow therapeutic range drug digoxin an R-value of >1.25 was considered clinically relevant.\textsuperscript{130}

\[
R = \frac{AUC_{+A}}{AUC} \tag{4}
\]

This thesis focuses on DDI involving inhibition of drug transport and therefore will drug A be referred to as the inhibitor (I) and drug B as the substrate. Reversal inhibition of drug transport can be competitive and noncompetitive. When the inhibition is competitive the binding site of the inhibitor and substrate are the same and for noncompetitive inhibition the binding sites differ.\textsuperscript{67, 131} The R-value of a competitive inhibition exhibiting single route clearance can be expressed according to Equation 5. At substrate concentrations well below the \(K_m\)-value, which is generally the case at clinical doses, DDI involving noncompetitive inhibition can also be described according to Equation 5.\textsuperscript{128}

\[
R = \frac{AUC_{+I}}{AUC} = 1 + \frac{I}{K_i} \tag{5}
\]

Preferable would \textit{in vivo} relevant DDI be predictable based on \textit{in vitro} studies and approaches to predict DDI have been developed. As the concentration of the inhibitor is a crucial parameter determining the degree of interaction Ito and co-workers have developed Equation 6 in order to estimate the unbound concentration of the inhibitor at the inlet of the liver \((I_{in,max,u})\) following oral dosing.\textsuperscript{131-133}

\[
I_{in,max,u} = \left( I_{max} + \frac{k \times \text{Dose} \times f_a (1 - E_G) }{Q_h} \right) \times f_u \tag{6}
\]

A better prediction of reversible CYP-mediated DDI at steady state was obtained using total maximum hepatic input concentration compared with maximum systemic concentration.\textsuperscript{134} Predictions of \textit{in vivo} DDI based on \textit{in vitro} data have proven to be complex and no clear consensus exists.\textsuperscript{134-137}

In a clinical situation multiple drug therapy is common and several drugs (and metabolites) may therefore contribute to the inhibition of the major elimination pathway.
1.2. Rosuvastatin

Statins competitively inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, an enzyme which mediates the conversion of HMG-CoA to mevalonate as a part of the biosynthesis of cholesterol in the liver. Reduced intracellular cholesterol causes an up-regulation of low-density-lipoprotein receptors on the cell surface, the increased number of cell receptors further enhances the clearance of cholesterol from the blood. Rosuvastatin (Crestor®) was approved by the US Food and Drug administration (FDA) in 2003 (Figure 5). Apart from rosuvastatin, there are seven statins, namely, atorvastatin, fluvastatin, pravastatin, cerivastatin, pitavastatin, lovastatin and simvastatin. Rosuvastatin is the most potent inhibitor of HMG-CoA reductase of the statins, partly explained by it having the highest number of interaction sites with the enzyme as shown by x-ray crystallography compared to the other statins.\textsuperscript{138, 139}

Figure 5. The chemical structure of rosuvastatin.

The first statin to be approved by US FDA was lovastatin, in 1987. The benefits of statin therapy in reducing the morbidity and mortality of patients with coronary heart disease were first studied in the Scandinavian heart study (4S) published in 1994.\textsuperscript{140} Since then, statins have been widely prescribed and, considering, the high prescription frequency, it is crucial to reduce the risk of the severe side-effects that are associated with statin therapy. In 2001, cerivastatin, which was approved by US FDA in 1997, was withdrawn from the market after several fatal cases of myopathy and
rhombomyolysis. The mechanisms underlying these severe side-effects are not fully understood, however there is a clear correlation between the risk of developing these side-effects and increased extra-hepatic exposure in humans. Certain single nucleotide polymorphisms (SNP) in the \( \text{SLCO1B1} \) gene have been shown to be associated with an increased risk of myopathy particularly at the higher statin doses. However, this correlation was not observed in rats, although it was concluded in the same investigation, that cerivastatin exhibited a higher degree of myotoxicity compared than rosuvastatin and simvastatin.

The hydrophilic nature of rosuvastatin predicts a low hepatic extraction, however involvement of multiple hepatic transport proteins result in an extensive hepatic distribution.\(^3\), \(^1\)\(^4\)\(^3\), \(^1\)\(^4\)\(^4\)

1.2.1. The sinusoidal transport of rosuvastatin

Numerous hepatic uptake transporters mediate the hepatic extraction of rosuvastatin in vitro, of these, OATP1B1 is considered to be the primary transporter involved.\(^3\) The OATP1B1-mediated transport of rosuvastatin has been observed in various in vitro systems and \( K_m \)-values ranging from 0.8 to 8.5 \( \mu \text{M} \) have been reported.\(^3\), \(^2\)\(^2\), \(^1\)\(^4\)\(^5\), \(^1\)\(^4\)\(^6\) Rosuvastatin is also a substrate of OATP1B3, OATP2B1 and OATP1A2. OATP1B3 and OATP2B1-mediated the transport of rosuvastatin in transfected HEK293 cells at \( K_m \)-values of 14.2±2.8 and 6.42±1.03 \( \mu \text{M} \), respectively.\(^3\) Lower \( K_m \)-values were reported in transfected HeLa cells, 9.8 and 2.4 \( \mu \text{M} \) for OATP1B3 and OATP2B1, respectively. Rosuvastatin was also a substrate for OATP1A2 in transfected HeLa cells (\( K_m \)-value of 2.6 \( \mu \text{M} \)).\(^2\)\(^2\) Ho and co-workers reported a sodium-dependant uptake of rosuvastatin in human hepatocytes of approximately 35%, suggesting NTCP-mediated transport of rosuvastatin (\( K_m \)-value of 65 \( \mu \text{M} \)). However, no sodium-dependant transport was observed in rat hepatocytes.\(^2\)\(^2\) OCT1 was not able to transport rosuvastatin in HeLa transfected cells.\(^2\)\(^2\)

In 2004, Simonson et al reported a 7.1 and 10.6-fold higher AUC and \( C_{\text{max}} \) in plasma following a single 20 mg rosuvastatin dose in heart transplant patients receiving an anti-rejection regimen that included cyclosporine. The interaction between rosuvastatin and cyclosporine was further investigated in OATP1B1 transfected Xenopus laevis oocytes and cyclosporine was found to competitively inhibit this transport (\( IC_{50} \)-value = 2.2±0.4 \( \mu \text{M} \)).\(^1\)\(^4\)\(^6\) A 1.88 and 2.21-fold increase in the plasma exposure and \( C_{\text{max}} \) of rosuvastatin were reported following co-administration with gemfibrozil for seven days in healthy volunteers.\(^1\)\(^4\) In vitro studies confirmed that gemfibrozil is an inhibitor of OATP1B1-mediated transport of rosuvastatin (\( IC_{50} \)-value
In addition, metabolites of gemfibrozil were also shown to inhibit the OATP1B1-mediated transport of pravastatin and cerivastatin.\textsuperscript{147,148}

Several studies have been conducted to evaluate the influence of genetic variations on the pharmacokinetics of rosuvastatin, they suggest that certain SNPs in the \textit{SLCO1B1} gene affect the plasma exposure of rosuvastatin.\textsuperscript{149-151} For instance, the plasma AUC and \(C_{\text{max}}\) in subjects with the c.521CC genotype were 1.65 and 1.79 times higher than in the wild-type c.521TT. No statistically significant effects of the pharmacokinetics of rosuvastatin were observed for the c.521TC genotype.\textsuperscript{151} Lee et al concluded that other factors than 521T>C or 388A>G polymorphism in \textit{SLCO1B1} were responsible for the ~2-fold increase in plasma exposure of rosuvastatin and its metabolites in Chinese, Malayan and Asian-Indian subjects compared to white subjects living in Singapore.\textsuperscript{152}

### 1.2.2. The canalicular transport of rosuvastatin

In the double transfected MDCKII cells expressing OATP1B1 and a canalicular transporter (MRP2, MDR1 or BCRP), Kitamura et al observed significant transport by all the efflux transporters studied.\textsuperscript{3} The ability of BCRP to transport rosuvastatin was also shown in BCRP-expressing membrane vesicles (\(K_{\text{m}}\) values of 10.8±1.1 \(\mu\text{M}\) and 307±89.4 \(\mu\text{M}\)).\textsuperscript{153} However, no transport of rosuvastatin was observed by MRP2 or MDR1 in single transfectant cell.\textsuperscript{153} The MDR1 inhibitor ketoconazole had no effect on the pharmacokinetics of a single oral dose of rosuvastatin in healthy volunteers.\textsuperscript{154}

Polymorphisms in genes of the canalicular transport proteins might shed a light on canalicular transporters importance for the disposition of rosuvastatin \textit{in vivo}. Zhang et al investigated the impact of the BCRP 421C>A polymorphism in Chinese males. In subjects with the 421CA or 421AA genotype, the plasma AUC and \(C_{\text{max}}\) were approximately 1.8 and 1.9 times higher than for the 421CC wild-type.\textsuperscript{155} Keskitalo et al investigated the effects of 421C>A polymorphism on the pharmacokinetics of rosuvastatin in the Finnish population. The 421AA genotype had a 2.44 and 2.31 times higher plasma AUC and \(C_{\text{max}}\) compared to 421CC, however no significant effects were detected for the 421CA genotype compared to the wild-type.\textsuperscript{156} In Bcrp\textsuperscript{-/-} knockout mice the biliary clearance of rosuvastatin was reduced by ~90% and plasma concentrations in the steady state increased a factor of 1.7.\textsuperscript{3} The importance of Mrp2 for the biliary excretion of rosuvastatin was observed as a 50% reduction of the biliary clearance of rosuvastatin in Eisai hyperbilirubinemic rats, which lack functional Mrp2, compared to Sprague-Dawley rats.\textsuperscript{3}
1.2.3. The metabolism of rosuvastatin

In vivo and in vitro studies both indicate that metabolism is of little importance in the disposition of rosuvastatin. Of an administered 20 mg oral dose of \([^{14}\text{C}]-\text{rosuvastatin}\) approximately 90% was recovered in the feces, of which 76.8% was in its unchanged form.\(^{157}\) The metabolites of rosuvastatin identified both in vivo and in vitro are rosuvastatin-lactone and N-desmethyl rosuvastatin.\(^{157, 158}\) Prueksaritanont et al also detected a \(\beta\)-1-\(O\)-acyl glucuronide conjugate following incubation with rosuvastatin in human hepatocytes.\(^{158}\) The formation of the glucuronide conjugate is considered to be mediated by UDP glucuronosyltransferase (UGT)1A1 and UGT1A3 in human hepatocytes.\(^{158}\)

The metabolism of rosuvastatin in vitro is low.\(^{159, 160}\) Fujino et al compared the metabolic clearance for several statins and rosuvastatin had the lowest clearance, with 1.1 \(\mu\text{l}
\cdot\text{min}^{-1}\cdot\text{mg}^{-1}\) protein, and fluvastatin the highest, with 33 \(\mu\text{l}
\cdot\text{min}^{-1}\cdot\text{mg}^{-1}\) protein.\(^{160}\) Rosuvastatin has been identified as a substrate for several cytochrome P450 (CYP) enzymes in vitro: CYP2C9, CYP2D6 and CYP3A4.\(^{158, 160}\) However, only a 1.14 and 1.09-fold increase in the plasma AUC and \(C_{\text{max}}\) of rosuvastatin was observed when rosuvastatin was co-administered with the potent CYP2C9 and CYP2C19 inhibitor, fluconazole in healthy volunteers.\(^{161}\) Itraconazole, the CYP3A4 and MDR1 inhibitor, increased the plasma exposure of 10 and 80 mg doses of rosuvastatin by a factor of 1.39 and 1.28, respectively.\(^{162}\) In addition, no DDI were observed between rosuvastatin and erythromycin (CYP3A4 inhibitor) or ketoconazole (CYP3A4 and MDR1 inhibitor).\(^{154, 163}\) Overall, the DDI associated with inhibition of the involved hepatic uptake transporters are considerably more marked and are likely to be of clinical relevance than the DDI arising from enzyme inhibition.
2. Aims of the thesis

The overall aims of this thesis can be summarized as being to:

- Investigate the biliary excretion and exposure of rosuvastatin in humans and pigs.

- Determine the importance of drug transport inhibition for the hepatobiliary disposition in vivo.

- Investigate the effect of concomitant administration of gemfibrozil, cyclosporine, rifampicin and imatinib on the hepatobiliary transport of rosuvastatin.

- Increase the understanding of the interplay between sinusoidal and canalicular transport proteins for the disposition of rosuvastatin.

- Develop and evaluate a non-invasive method with which to investigate the biliary excretion in healthy volunteers.
3. Methods

Rosuvastatin was chosen as a model drug to investigate hepatobiliary transport processes \textit{in vivo} in humans and pigs owing to its proven dependency on transport proteins.

3.1. Investigation of biliary excretion in humans

In Papers I and II an intestinal intubation catheter, the Loc-I-Gut catheter, was used to sample bile from the distal duodenum/proximal jejunum in healthy volunteers. The catheter, which is composed of six separated channels, is introduced orally and has a tungsten weights at its tip to facilitate the passage into the intestine via the stomach (Figure 6). It measures 175 cm, with an external diameter of 5.3 mm. Two of the Loc-I-Gut channels are coupled to inflatable balloons and, when both are inflated with 25-30 ml air, a 10 cm long isolated segment is created between them in the jejunum.\textsuperscript{164} The Loc-I-Gut catheter was developed in the late ‘80-ties and, since then, has been used to investigate permeability and the dissolution of drugs in the human intestine \textit{in vivo}.\textsuperscript{164, 165} Its applicability as a method to study biliary excretion of drugs was investigated in Paper I.

In Papers I and II, the biliary excretion of a single intrajejunal rosuvastatin dose was investigated. The Loc-I-Gut catheter was positioned in the distal duodenum/proximal jejunum, below the Papilla of Vater, where bile empties into the small intestine. The drug administration and biliary sampling sites were separated by the inflated proximal balloon of the Loc-I-Gut catheter (Figure 6). Bile was sampled every 20 minutes using a vacuum pump for 240 and 200 minutes in Papers I and II, respectively. Blood samples were withdrawn simultaneously with the bile samples to investigate the concurrent plasma and bile concentration time profiles. The fraction of the administered oral dose excreted into bile and the biliary clearance were calculated according to Equations 7 and 8, respectively.
Figure 6. The Loc-I-Gut catheter positioned in the distal duodenum/proximal jejunum was used to collect duodenal bile in healthy volunteers in Papers I and II.

\[ f_{e,\text{bile}} = \frac{\sum A_e_{\text{bile}}}{\text{Dose}} \]  
(7)

\[ \text{CL}_{\text{bile}} = \frac{\sum A_e_{\text{bile0-t}}}{\text{AUC}_{\text{plasma0-t}}} \]  
(8)

3.2. Investigation of hepatobiliary transport in pigs

In the porcine model, bile is sampled directly from the common hepatic duct by placing a catheter in the common hepatic duct for six hours.\textsuperscript{115} The direct measurement of the in vivo hepatic extraction (Equation 9) was enabled by the placement of catheters in the hepatic and portal veins, respectively. The catheter in the hepatic vein (VH) was positioned using fluoroscopy and verified using contrast injection. The catheter was placed in the portal vein (VP) via the superior mesenteric vein. Another catheter was placed in the right femoral vein (VF) to monitor the peripheral plasma exposure of the investigated drug. The Loc-I-Gut catheter was placed in the distal
duodenum/proximal jejunum by making an incision in the upper part of the duodenum. In Paper II the balloons of the Loc-I-Gut catheter were inflated with 20-25 ml air and ligated, distally from the distal balloon and proximal to the proximal balloon. This created a 10 cm long isolated segment, which was perfused at 2 ml/min for 100 minutes with phosphate buffer, enabling the intestinal secretion of drugs to be investigated (Equation 10).

$$E_H = \frac{\text{AUC}_{\text{VP0-t}} - \text{AUC}_{\text{VH0-t}}}{\text{AUC}_{\text{VP0-t}}}$$ (9)

$$\text{CL}_{\text{int estine}} = \frac{\sum \text{Ae}_{\text{int estine30–100}}}{\text{AUC}_{\text{VF30–100}}}$$ (10)

3.3. Inhibition of drug transporters in vivo

Inhibiting a specific transport protein in vivo is challenging. The following inhibitors were chosen for the investigations reported in Papers II-IV: gemfibrozil, cyclosporine, rifampicin and imatinib.

Gemfibrozil was chosen as a OATP1B1 inhibitor in Papers II and III as it inhibited the OATP1B1-mediated transport of rosuvastatin in OATP1B1 expressing Xenopus laevis oocytes at a IC\textsubscript{50}-value of 4.0±1.3 μM.\textsuperscript{145} Ho et al reported an IC\textsubscript{50}-value of 25 μM using OATP1B1-expressing HeLa-cells.\textsuperscript{22} Gemfibrozil is a lipid lowering agent hence the DDI with rosuvastatin is of clinical relevance, because a multidrug dosing regime can be applied for the treatment of complex hyperlipidemia.\textsuperscript{145} Gemfibrozil was also able to inhibit the transport of rosuvastatin by other transporters, at reported IC\textsubscript{50}-values of 8 and 23 μM for OATP2B1 and NTCP, respectively.\textsuperscript{22} It is possible that the OATP1B3-mediated uptake of rosuvastatin was affected by gemfibrozil as high concentrations of gemfibrozil (200 μM) reduced the uptake of fluvastatin into OATP1B3 transfected HEK293 cells by 62%. Surprisingly, 200 μM of gemfibrozil reduced the uptake of fluvastatin in human hepatocytes by only 27%.\textsuperscript{166} The gemfibrozil-1-O-glucuronide was a more potent inhibitor than the parent compound of the OATP1B1-mediated uptake of cerivastatin in transfected MDCKII cells, as revealed by the lower IC\textsubscript{50}-values reported for the glucuronide compared to the parent compound, 24.4±19.8 and 72.4±28.4 μM, respectively.\textsuperscript{148} No reports have confirmed
inhibitory action of gemfibrozil on canalicular transport proteins such as MRP2 and MDR1.\textsuperscript{71,147}

An extensive DDI involving rosuvastatin and cyclosporine was reported by Simonson and co-workers in 2004.\textsuperscript{146} Based on this study, the effects of a two-hour-long intravenous infusion of cyclosporine on the pharmacokinetics of rosuvastatin in plasma and bile were investigated in Paper III. Both the sinusoidal and canalicular transport proteins involved in the hepatobiliary disposition of rosuvastatin are inhibited by cyclosporine, as it inhibits the OATP1B1-mediated transport of rosuvastatin in transfected \textit{Xenopus laevis} oocytes at an IC\textsubscript{50}-value of 2.2±0.4 μM.\textsuperscript{146} Furthermore, IC\textsubscript{50}-values of the OATP1B1, OATP2B1, OATP1B3 and NTCP-mediated transport of rosuvastatin of 0.31, 0.07, 0.06, 0.37 μM, respectively, have been reported using transfected HeLa cells.\textsuperscript{22} In addition, the canalicular transporters, BCRP, MDR1 and MRP2, have also been reported to be inhibited by cyclosporine.\textsuperscript{70, 167-169} A intravenous dose of cyclosporine was chosen in Paper III because of its high and variable first pass metabolism in humans and pigs.\textsuperscript{170-172}

In Paper IV, rifampicin was chosen as an inhibitor of the sinusoidal transport of rosuvastatin. According to several studies using various substrates and \textit{in vitro} models, rifampicin is an inhibitor of OATP1B1 and OATP1B3.\textsuperscript{173-176} K\textsubscript{i}-values ranging from 3 to 17 μM and 1.6 to 5 μM have been reported for inhibition of OATP1B1 and OATP1B3, respectively.\textsuperscript{173-175} In OATP1B1 transfected HEK293 cells, rifampicin reduced the cellular uptake of atorvastatin at a K\textsubscript{i}-value of 3.08±0.49 μM.\textsuperscript{174} Furthermore, the Ntcp-mediated transport of Taurocholate transport was reduced by 35% following co-incubation with 100 μM rifampicin in transfected \textit{Xenopus laevis} oocytes.\textsuperscript{177} Besides inhibiting sinusoidal transporters, rifampicin inhibited the canalicular transporters MRP2 and BSEP.\textsuperscript{178-181} However, no effect by rifampicin (50 μM) were observed on the MDR1-mediated transport of atorvastatin in MDR1-overexpressing M-MDR1 cells.\textsuperscript{179}

Imatinib was administered as a canalicular transport inhibitor in Paper IV because it is a known BCRP inhibitor.\textsuperscript{182-186} The accumulation of topotecan was significantly increased following co-incubation with 1 μM imatinib in BCRP transfected Saos2 cells.\textsuperscript{184} Moreover, moderate inhibition (of 26%) by imatinib was observed in MRP2-mediated transport of estradiol-17β-D-glucuronide into MRP2-overexpressing inverted membrane vesicles.\textsuperscript{187} In addition, imatinib inhibited MDR1-mediated transport of calcein acetoxymethyl ester at K\textsubscript{i}-value of 18.3 μM.\textsuperscript{70}
3.4. Analytical methods

The analysis of rosuvastatin and gemfibrozil in plasma and bile was conducted using an High-performance liquid chromatography (HPLC) coupled to a Quattro LC quadrupole-hexapole-quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ionization interface. Rosuvastatin was monitored in a positive mode and gemfibrozil in a negative mode. In Paper I, atorvastatin was used as an internal standard for rosuvastatin, however in Papers II-IV, trideuterated rosuvastatin was applied as the internal standard. Hexadeuterated gemfibrozil was the internal standard for gemfibrozil in Papers II and III.

The concentration of eleven bile acids in the collected duodenal bile in Paper I were analyzed with HPLC, using an evaporated light scattering detector. The internal standard for the bile acid analysis was nor-deoxycholic acid.

In Paper III, cyclosporine in whole blood was analysed using the CEDIA® Ciklosporin PLUS-assay (Microgenics Corp., Fremont, CA, US). The immunoassay was performed by the Department of Clinical Chemistry and Pharmacology at the University Hospital in Uppsala, Sweden.

3.5. Pharmacokinetic data analysis

Pharmacokinetic parameters were analyzed using non-compartmental methods (WinNonlin Version 4.0, Pharsight Corp., Mountain View, CA, USA). The terminal rate constant ($\lambda_z$) was determined by logarithmic-linear regression analysis of the last three to five concentration time points. The area under the curve (AUC) in plasma were calculated using the linear/logarithmic trapezoidal rule. In bile, multiple peaks were observed and, because of this, the AUC in the bile were calculated using the linear trapezoidal rule for the ascending areas and the logarithmic trapezoidal rule for the descending ones.

3.6. Statistical analysis

All data are presented as arithmetic means ± one standard deviation, unless stated otherwise. $C_{\text{max}}$ and the AUC were logarithmically transformed before the statistical analysis was conducted. The statistical analysis was performed using Minitab 14 (Minitab Inc., PA, USA) and the Students t-test. Comparisons of the degree of an investigated interaction are expressed as the ratio of the geometric means and variability, are presented as the 95% confidence interval (CI).
4. Results and discussion

The accumulation of rosuvastatin in bile was extensive compared to plasma, with $\text{AUC}_{\text{bile}}/\text{AUC}_{\text{plasma}}$ ratios of 617 (315-1198) and 1700 (376-11300) in humans and pigs, respectively. In contrast, the biliary accumulation of gemfibrozil was minor, with $\text{AUC}_{\text{bile}}/\text{AUC}_{\text{plasma}}$ ratios of 1.33 (0.02-28.3) and 0.06 (0.03-0.07) in humans and pigs, respectively. The simultaneous measurement of the concentration of rosuvastatin in the portal and hepatic vein compartment enables a direct determination to be made of the hepatic extraction in vivo in pig. As expected for a drug compound that is extensively distributed in the hepatobiliary system, the extraction determined using Equation 9 was higher than that determined as the ratio of the hepatic clearance over the hepatic blood flow. In pigs, the hepatic extraction of rosuvastatin was 0.82±0.16, compared to an estimated hepatic extraction in humans of 0.63.\(^4\) In an attempt to elucidate the importance of hepatic transporters, and particularly of the sinusoidal versus the canalicular membrane transporters, the following transport inhibitors were administered individually with rosuvastatin: gemfibrozil, cyclosporine, rifampicin and imatinib. An overview of the dosing regimen and the administration sites is given in Table 2. The capacity of the liver to extract rosuvastatin from the blood was high, and was dependant on hepatic drug transporters (Figure 7).

Table 2. A summary of the dosing regime in Papers I-IV.

<table>
<thead>
<tr>
<th>Paper</th>
<th>N</th>
<th>Species</th>
<th>Rosuvastatin</th>
<th>Co-administrant</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>8 (10)</td>
<td>H</td>
<td>10 mg, i.j.</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>8 (11)</td>
<td>H</td>
<td>20 mg, i.j.</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>8 (11)</td>
<td>H</td>
<td>20 mg, i.j.</td>
<td>gemfibrozil; 600 mg, i.j.</td>
</tr>
<tr>
<td>III+IV</td>
<td>6+2</td>
<td>P</td>
<td>80 mg, i.j.</td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>4</td>
<td>P</td>
<td>5.9 mg, i.v.</td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>6</td>
<td>P</td>
<td>80 mg, i.j.</td>
<td>cyclosporine; 300 mg, i.v. infusion</td>
</tr>
<tr>
<td>III</td>
<td>6</td>
<td>P</td>
<td>80 mg, i.j.</td>
<td>gemfibrozil; 600 mg, i.j.</td>
</tr>
<tr>
<td>IV</td>
<td>6</td>
<td>P</td>
<td>80 mg, i.j.</td>
<td>rifampicin; 20 mg/kg, i.v. infusion</td>
</tr>
<tr>
<td>IV</td>
<td>6</td>
<td>P</td>
<td>80 mg, i.j.</td>
<td>imatinib; 14 mg/kg, iv infusion</td>
</tr>
</tbody>
</table>

N: number of subjects that completed the study (number of subjects included in the study), H: human, P: pig, i.j.: intrajejunal, i.v.: intravenous
Figure 7 The concentration time profiles of rosuvastatin in the portal (VP) and hepatic (VH) vein compartments in pig are presented in Figure 7A and 7B, respectively. Rosuvastatin (80 mg) was administered as a single intrajejunal (i.j.) dose alone (n=8) or together with a transport inhibitor (n=6): gemfibrozil (GFZ), cyclosporine (CsA), rifampicin (RIF) and imatinib (IM). Gemfibrozil was administered as an i.j. dose 20 minutes prior to the rosuvastatin dose, whereas rifampicin, imatinib and cyclosporine were administered as a two-hour-long intravenous infusion staring one hour prior to giving the rosuvastatin dose. The data are presented as arithmetic means values and the variability as the SEM.
4.1. Reduction of the hepatic extraction of rosuvastatin by cyclosporine and rifampicin

Significant increases in the plasma exposure of rosuvastatin in the hepatic vein compartment were observed when cyclosporine and rifampicin were administered as intravenous infusions in Papers III and IV (Figure 7). In Paper III, cyclosporine increased the AUC and C$_{\text{max}}$ in the hepatic vein by factors of 9.1 (95% CI: 3.2-25.9, $p<0.001$) and 16.0 (95%CI: 5.4-47.2, $p<0.001$), respectively. However, the AUC and C$_{\text{max}}$ in the portal vein increased by a more modest extent by a factors of 1.6 (95%CI: 1.0-2.6, $p<0.05$) and 2.0 (95% CI: 1.2-3.3, $p<0.05$), respectively. In Paper IV, rifampicin increased the AUC and C$_{\text{max}}$ in the hepatic vein compartment by factors of 6.3 (95%CI: 3.1, 32.1, $p<0.01$) and 10 (95%CI: 1.9, 21.3, $p<0.01$), respectively. However, no significant difference was observed in the AUC in the portal vein, although C$_{\text{max}}$ increased 2.7-fold (95%CI: 1.4-5.6, $p<0.01$). The hepatic vein compartments increased by a considerably higher degree compared to the portal vein compartments, indicating that the DDI were located predominantly in the liver and not the intestine.

The inhibitory action of cyclosporine and rifampicin was direct, and coincided with the highest exposure to the inhibitor, as shown for cyclosporine in Figure 8. Multiple transport inhibitors can be affected by cyclosporine and rifampicin. In Paper III, the maximum unbound concentration of the cyclosporine in the portal vein (C$_{\text{max \, u}}$) was approximately 0.38 $\mu$M, which is above the reported IC$_{50}$-value by Ho et al, of 0.31 $\mu$M, but below the value reported by Simonson et al of 4.0±1.3 $\mu$M. The C$_{\text{max \, u}}$ of cyclosporine in the portal vein in Paper III was also sufficient to inhibit the OATP1B3-, OATP2B1- and NTCP-mediated transport of rosuvastatin according to their reported IC$_{50}$-values. In Paper III, the analysis of cyclosporine in whole blood was performed using the immunoassay technique. Compared to specific HPLC analysis, cross-reactivity of cyclosporine metabolites with the cyclosporine antibody will result in approximately 40% higher concentrations using the CEDIA® Ciklosporin PLUS-assay compared to HPLC. In Paper III, cyclosporine could have inhibited the canalicular transport proteins, MRP2, MDR1 and BCRP. However, the ~50% reduction observed in both the biliary exposure and biliary clearance is in line with the ~50% reduction in the hepatic extraction, supporting the conclusion that, sinusoidal uptake transporters were preferentially inhibited by cyclosporine. The significant increase of the plasma exposure by cyclosporine in relation to the 50% reduction of the biliary exposure is in agreement with inhibition of sinusoidal drug transport according to the physiologically based pharmacokinetic (PBPK) model developed by Watanabe et al.
Figure 8. The impact of cyclosporine (CsA, 300 mg) on the plasma exposure of a single intrajejunal dose of 80 mg rosuvastatin (RV). Cyclosporine was administered as a two-hour-long intravenous infusion, the rosuvastatin dose was administered one hour from the start of the cyclosporine infusion. The concentration time profiles for rosuvastatin and cyclosporine were monitored in the portal (VP) and hepatic (VH) vein compartments for up to 300 minutes after the administration of the RV dose.

Rifampicin reduced the hepatic extraction of rosuvastatin by 35% \( (p<0.01) \) and this was reflected by a 44% reduction of the biliary exposure \( (p<0.05) \). The absence of a significant increase in the AUC\textsubscript{VP} supports the conclusion that the effects of rifampicin were associated with inhibition of hepatobiliary transport proteins rather than the intestinal transporters. Furthermore, the considerable increase observed in the hepatic vein compartment compared to the reduction of the biliary exposure by rifampicin corresponded to inhibition of sinusoidal transport in accordance with the PBPK model by Watanabe and coworkers.\textsuperscript{191}

4.2. The effects of an single oral dose of gemfibrozil on the pharmacokinetics of rosuvastatin

The absence of a gemfibrozil-related affect on the pharmacokinetics of rosuvastatin in both humans and pigs in Papers II and III was attributed of low exposure of the inhibitor (Figure 9). In Paper II, the maximum unbound plasma concentration of a 600 mg intrajejunal dose of gemfibrozil at the inlet of the human liver was estimated to be 0.58 \( \mu \text{M} \), using Equation 6. In Paper III, the same dose and administration site in pigs resulted in a similar \( I_{\text{in max, u}} \) as in humans (i.e. 0.55 \( \mu \text{M} \), in the portal vein compartment). At the inlet of the liver in both humans and pigs the unbound concentration of the inhibitor was well below the IC\textsubscript{50}-values of 4.0±1.3 \( \mu \text{M} \) and 25 \( \mu \text{M} \) reported
for the inhibition of OATP1B1-mediated transport of rosuvastatin by gemfibrozil (Figure 9).\textsuperscript{22, 145} Interestingly, an estimated $I_{\text{in max}}$ of 0.84 μM in humans administered twice daily with 600 mg gemfibrozil for seven days increased the plasma AUC and $C_{\text{max}}$ of a single rosuvastatin dose by 1.88 and 2.21-fold, respectively.\textsuperscript{145} The differences between single and repeated dosing of gemfibrozil might be attributed to the accumulation of metabolites. Gemfibrozil is highly metabolized and the major metabolite is gemfibrozil 1-O-β-glucuronide, which is a more inhibitor of the OATP1B1-mediated transport of cerivastatin and pravastatin than the parent compound \textit{in vitro}.\textsuperscript{147, 148, 192} Based on the chemical reactivity and the likely formation-rate dependent half-life of the glucuronide metabolite, it is likely that other metabolites explain these observed differences.\textsuperscript{193, 194}

Figure 9. The concentration time profiles of gemfibrozil in plasma and bile following a 600 mg intrajejunal dose in humans (A) and pigs (B). Both the total ($C_{\text{tot}}$) and unbound concentrations ($C_{u\beta}$=0.9935) of gemfibrozil in plasma are presented. The data are presented as arithmetic means and the variability is expressed as the SEM (Figure A, n=8; Figure B, n=6).
4.3. The intestinal absorption of rosuvastatin *in vivo*

The fraction of the rosuvastatin dose absorbed was higher in pigs than in humans, 0.92 and 0.54, respectively (Equation 1). The apparent permeability of rosuvastatin in MDR1-MDCKII cells was low, ~8 nm/s, and suggested no involvement of MDR1-mediated efflux of rosuvastatin. The physiochemical properties of rosuvastatin predict low passive transcellular diffusion (clog $D_{\text{pH 7.25 °C}}$ -2.29, SciFinder® database; log D of -0.33, Chapman and MacTaggart). Intestinal influx transporters might be involved in facilitating the absorption of rosuvastatin from the small intestine. OATP1A2 and OATP2B1 have been detected in intestinal tissue samples. In 2007, Glaeser et al reported the presence of OATP1B1 mRNA in human intestinal biopsies, though to my knowledge no OATP1B1 protein has ever been detected in the small intestine. In Papers II and III, the absence of an effect on the pharmacokinetics of rosuvastatin of an intrajejunal bolus dose of 600 mg gemfibrozil in humans and pigs indicate that functional intestinal OATP1B1 are absent *in vivo* (Figure 7). Intestinal OATP2B1 is considered to be involved in the oral absorption of pravastatin. The importance of intestinal OATP2B1 is supported by its reported pH-dependent transport capacity. The uptake of estrone-3-sulfate into OATP2B1 transfected HEK293 cells was higher at pH 5.5 than at pH 7.4. Gemfibrozil inhibited the OATP2B1-mediated transport of fluvastatin in transfected HEK293 cells by ~40% and ~70% at 100 and 200 μM. In contrast to an inhibition of intestinal OATP, gemfibrozil was found to increase the partial plasma exposure of rosuvastatin from drug administration and 200 minutes forward by a factor of 1.56 (95%CI: 0.74-1.57, $p<0.05$) in Paper II. Altogether, the absence of a significant gemfibrozil-induced effect on the pharmacokinetics of rosuvastatin in Papers II and III suggests that intestinal OATPs are of little importance for the intestinal absorption of rosuvastatin *in vivo*.

Intestinal excretion of rosuvastatin was observed and affected by gemfibrozil in Paper III. This might suggest involvement of intestinal efflux transporters in the disposition of rosuvastatin. Initial inhibition of an intestinal efflux transporter might explain the increase in partial plasma exposure observed in Paper II from 0 to 200 minutes post-dose. However, the estimated fraction absorbed, 0.92 in Paper III, does indicate that the efflux transporters are of minor importance to the overall pharmacokinetics of rosuvastatin. Intestinal efflux transports known to mediate the transport of rosuvastatin are BCRP and MRP2. The mRNA expression of MRP2 and BCRP in human jejunal tissue samples has been revealed by Hilgendorf et al to be higher than that of MDR1. MDR1 mediated the transport of rosuvastatin in OATP1B1/MDR1 double transfectant MDCKII cells, however, no transport were observed in single transfectants.
Furthermore, oral administration of the MDR1 inhibitor, ketoconazole, had no effect on the pharmacokinetics of an 80 mg oral dose of rosuvastatin.\textsuperscript{154}

Inhibition of intestinal efflux transport might explain the increased intestinal excretion of rosuvastatin by gemfibrozil in Paper III. Further studies are needed to investigate whether gemfibrozil and/or its metabolites inhibit BCRP and MRP2-mediated transport of rosuvastatin. No inhibition of MRP2-mediated uptake of pravastatin by gemfibrozil or its metabolites was observed by Nakagomi-Hagihara et al in MRP2-expressing membrane vesicles.\textsuperscript{147} Additionally, gemfibrozil did not inhibit the MRP2- or MDR1-mediated transport in MRP2-expressing S9 membrane vesicles or MDR1 overexpressing cells as revealed by Yamazaki et al in 2005.\textsuperscript{71}

![Figure 10](image.png)

*Figure 10.* The accumulated amount of rosuvastatin excreted into bile during a period of 300 minutes. Rosuvastatin (80 mg) was administered into the jejunum using the Loc-I-Gut catheter alone or together with imatinib (14 mg/kg). Imatinib was administered as a two hour intravenous infusion, starting one hour prior to administering the rosuvastatin dose. The data are presented as arithmetic means and the variability is expressed as SEM (n=6).

Minor effects on the intestinal absorption of rosuvastatin were observed in Paper IV when rosuvastatin was co-administered with imatinib, a selective BCRP inhibitor.\textsuperscript{182-186} Rosuvastatin was administered as an intrajejunal bolus dose one hour after the initiation of a two-hour-long intravenous infusion of imatinib. No significant effects were evident on the plasma AUC of rosuvastatin in either the portal or hepatic vein compartment, respectively (Figure 7). Instead, $C_{\text{max}}$ increased and a tendency towards increased biliary excretion of rosuvastatin was observed (Figure 10). The increased biliary
excretion of rosuvastatin appeared to occur during the hour immediately following the administration of rosuvastatin, i.e., concurrently with the duration of the imatinib infusion and coinciding with the increase in plasma $C_{\text{max}}$ (Figure 10). The alterations in the plasma compartments were reflected in the biliary compartment as the $T_{\text{max}}$ in bile was reduced and a tendency towards a higher $C_{\text{max}}$ in bile was noted (Figure 7). These observations might suggest an increased intestinal absorption rate. Nevertheless, $C_{\text{max}}$ in the hepatic vein compartment exhibited a higher increase to that in the portal vein compartment; the values were 4.3-fold (95%CI: 1.4-12.9; $p<0.05$) and 1.9-fold (95%CI: 1.2-2.9; $p<0.01$), respectively. This suggests that imatinib had an impact on the disposition of rosuvastatin in the liver. On the other hand, the fact that the biliary excretion and $E_{\text{H}}$ were unaffected indicates that the effects on the liver were temporary (Paper IV).

4.2. Investigation of biliary excretion in healthy volunteers using the Loc-I-Gut catheter

In Paper I, the applicability of the Loc-I-Gut catheter as a tool for collecting duodenal bile in healthy volunteers was investigated. Studies performed prior to this investigation had indicated that rosuvastatin underwent enterohepatic circulation owing to the occurrence of multiple peaks in its plasma concentration time profiles. In Paper I, 11.5% (2.19%-16.0%) of the 10 mg intrajejunally administered dose was recovered in the duodenal bile. The biliary concentration of rosuvastatin was significantly higher than in plasma at all time-points. A considerable biliary accumulation of rosuvastatin in vivo was also observed in Paper II following a 20 mg dose of rosuvastatin being administered into the jejunum in healthy volunteers using the Loc-I-Gut catheter. However, in Paper II, the fraction of the administered dose recovered in bile was a mere 0.8% (0.7%-2.3%). A likely explanation for the differences in dose recovery between Papers I and II is the 40 minute shorter bile sampling period in the second paper. The biliary flow is dependant on MMC and was highly variable in Papers I and II. The biliary flow in Paper I is presented in Figure 11. The high variability associated with biliary flow is in accordance with previous reports. Consequently, a reduction of the bile sampling period will greatly affect the fraction of the dose recovered in bile. In Paper II, $^{14}$C-PEG4000 was included as a marker substance in order to monitor any leakage between the drug administration and the bile collection sites. This had not been done in the first paper. In Paper II, one subject was excluded from the investigation because of continuous leakage. Only four additional bile samples were contaminated in the remaining eight
subjects of the 123 samples collected, and the exclusion of these four samples did not affect the outcome of the study. Previous studies using the Loc-I-Gut catheter with a semi-open segment design report a low occurrence of contamination.\textsuperscript{196-199} It is unlikely that a considerable leakage occurred in Paper I, which would explain the over 14 times higher fraction recovered in bile in Paper I than in Paper II. The shorter bile collection period and considerable interindividual variability are more plausible explanations for the observed differences.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{biliary_flow_graph.png}
\caption{The biliary flow in healthy volunteers following the administration of a 10 mg rosuvastatin dose into the jejunum using the Loc-I-Gut catheter.}
\end{figure}

Collection of duodenal bile in healthy volunteers has its limitations and it is difficult to overcome the interindividual variability of gall bladder contraction and biliary flow patterns. By incorporating a gamma scintigraphic camera and gall bladder contraction stimulants with intestinal intubation, the variability can be reduced by enabling correction of the fraction of the dose remaining in the gall bladder at the end of the experiment. However, quantitative measurements of biliary excretion in healthy volunteers can only be performed on drugs with a short half-life (less than two hours) because of the limited bile collection time. Nevertheless, the bile collection technique used in Papers I and II is a more simple method with which to investigate biliary excretion, and it can be used in an early phase of drug development, to estimate the biliary clearance and to detect bile specific metabolites.\textsuperscript{200, 201}
5. Conclusions and future outlook

The main objective of this thesis was to study the involvement of hepatic transport proteins in the hepatobiliary disposition in vivo. Rosuvastatin was chosen as the investigated drug as its hepatic disposition is reported to be dependent on transport processes. From the investigations presented in this thesis, it can be concluded that:

- Rosuvastatin was significantly accumulated in bile both in humans and pigs. The hepatic extraction in humans was reported to be 0.63 however using the porcine model the direct hepatic extraction in vivo was considerably higher >0.8. This indicates efficacy of the enterohepatic circulation in optimizing the hepatic exposure, and hence in directing rosuvastatin to its site of action.

- The dependence of hepatic transport proteins on the disposition of rosuvastatin in vivo was observed in Papers III and IV, as the transport inhibitors, cyclosporine and rifampicin, significantly altered the pharmacokinetics of rosuvastatin. The highest impact of cyclosporine and rifampicin was observed in the hepatic vein compartments, rather than the portal vein compartments, which indicates that the DDI was located to the liver rather than the intestine. These DDI were considered to predominantly be related to inhibition of sinusoidal transport rather than canalicular transport in accordance with a previously reported PBPK model by Watanabe and co-workers.

- The BCRP inhibitor, imatinib, did not affect the plasma and biliary exposure of rosuvastatin in Paper IV. The absence of an effect by imatinib on the plasma exposure of rosuvastatin suggests that the intracellular concentration of the inhibitor in the hepatocyte was insufficient to affect the canalicular transport of rosuvastatin, or that the inhibition of BCRP by imatinib in vivo was not sufficiently potent.

- Gemfibrozil administered as a single intrajejunal dose did not affect the hepatobiliary transport of rosuvastatin in humans or in pigs. In a previous study, when gemfibrozil was administered repeatedly, significant increase of the plasma pharmacokinetics of rosuvastatin were observed. A plausible explanation for the observed
difference between single and repeated dosing is the accumulation of metabolites that are able to affect the disposition of rosuvastatin following repeated dosing.

- An intestinal excretion of rosuvastatin in pigs was observed in Paper III, and gemfibrozil increased the intestinal excretion of rosuvastatin. These observations suggest a minor involvement of intestinal efflux transporters that is susceptible to inhibition by gemfibrozil, however, which efflux transporter is involved needs further investigation.

- Application of the Loc-I-Gut catheter for investigation of biliary excretion of drugs can be useful when assessing the accumulation ratio of rosuvastatin in bile compared to plasma and to determine bile specific metabolites. The monitoring of gall bladder emptying is recommended to obtain a quantitative measurement of the fraction excreted into bile. Nevertheless, quantitative bile excretion studies are limited to drugs with short elimination half-lives (t_{1/2} less than two hours). A major advantage of this method is that it is not associated with discomfort for the subjects. The Loc-I-Gut catheter is positioned in the jejunum within 1-1.5 h, with at a high rate of success (87%). The porcine model offers an sampling from multiple compartments and enables a direct measurement to be made of the intestinal absorption, the hepatic extraction and biliary excretion.

*In vivo* studies of hepatobiliary disposition are challenging. The hepatobiliary system is complex, as its physiological localization, the complex cellular structure involving numerous transporters and enzymes, and the interindividual variability in protein expression and biliary flow all affect the *in vivo* disposition of a drug under investigation. The investigations presented here conclude that hepatic transport proteins are important for the hepatobiliary disposition of rosuvastatin *in vivo*. Inhibition of hepatic uptake transporters can result in considerable increases in the plasma exposure of rosuvastatin. The Loc-I-Gut catheter can be utilized for the investigation of biliary accumulation and to determine bile specific metabolites, however, it has limitations regarding the reliability of the quantitative measurements obtained. The porcine model enables the collection of hepatic bile for up to six hours and offers a possibility to determine the direct hepatic extraction *in vivo*. 
9. Populärvetenskaplig sammanfattning


Fokus för avhandlingen har varit att studera hur rosuvastatin fördelas i kroppen, framför allt i levern, och vilka processer som styr denna fördelning. Rosuvastatin är ett läkemedel som ges vid förhöjda blodfetter genom att minska bildandet av kolesterol i levercellen. Eftersom rosuvastatin utövar sin farmakologiska effekt inuti levern, är det önskvärt att en stor andel av rosuvastatin i blodet tas upp av cellerna i levern. En cell omges av ett cellmembran bestående av lipider, d v s fett. För att en molekyl ska tas upp av cellen, behöver den passera detta membran av fett. För molekyler som i sig är ”feta”, är detta inget problem, utan de kan fördela sig i membranet och på så sätt ta sig in i cellen (enligt lika- löser likaprincipen). För ”vattenlika” molekyler sker denna fördelning mycket långsamt. De kan istället binda till ämnen, transportproteiner, på cellmembranet, varpå de transporteras in i cellen. Det finns över 400 olika transportproteiner i kroppen och levercellen har flera typer av transportproteiner på sitt cellmembran (Figur 4). Rosuvastatin är mer ”vattenlik” än ”fet” men trots det fann vi i artikel III att över 80% av de i blodet passerande molekylerna togs upp av levern i gris. I människa har det tidigare rapporterats att 63% av molekylerna tas upp.

Det effektiva leverupptaget av rosuvastatin kan förklaras av att det kan binda till flera av de transportproteiner som finns på levercellens membran. Efter det att rosuvastatin utövat sin farmakologiska effekt i levern binder det till transportproteiner på levercellens andra sida (Figur 4). Dessa proteiner transporterar rosuvastatin ut i gallan som så småningom töms i tunntarmen. Utsöndring via galla är ett sätt för kroppen att göra sig av med ämnen. Galla består bland annat av gallsalter, vars uppgift i kroppen är att underlåta att fett från maten absorberas. Rosuvastatinmolekyler som tömts i tunntarmen via gallan kan återabsorberas och återigen utöva sin farmakologiska effekt i
levern. Denna recirkulation utav en molekyl kallas även för enterohepatisk cirkulation och gör att leverns exponering för rosuvastatin maximeras och därmed även dess farmakologiska effekt. I de arbeten som ligger till grund för denna avhandling har betydelsen och samspelet mellan dessa transportproteiner studerats.

Samtidigt intag av ett annat läkemedel kan påverka effektiviteten hos dessa transportproteiner och ge upphov till en läkemedelsinteraktion. En orsak till en läkemedelsinteraktion kan vara att transportproteinernas kapacitet att transportera rosuvastatin hämmas, vilket resulterar i att mindre rosuvastatin tas upp av levern och att den andel som når systemkretsloppet ökar. En ökad exponering av rosuvastatin i systemkretsloppet är inte önskvärt då det är associerat med ökad risk för allvarliga biverkningar. Av den anledningen är det av betydelse att studera dessa läkemedelsinteraktioner och få kunskap om vad som styr dessa processer.

Fyra läkemedelsinteraktioner har studerats i delarbete I-IV. I delarbete I utvecklades en metod för att studera gallutsöndring i friska frivilliga personer. En slang fördes ner i tunntarmen via munnen och positionerades så att den galla som tömdes ut i tunntarmen kunde samlas. I delarbete II och III studerades läkemedelsinteraktionen mellan rosuvastatin och gemfibrozil i friska frivilliga personer och i grisar. Vidare studerades interaktionen mellan rosuvastatin och ciklosporin, rifampicin och imatinib i grisar. Av dessa interaktioner orsakade ciklosporin och rifampicin signifikant ökning av den systemiska exponeringen av rosuvastatin genom att hämma de transportproteiner som ansvarar för att ta extrahera rosuvastatin från blodet in i levern. Gemfibrozil påverkade inte plasmakoncentrationen av rosuvastatin nämnvärt på grund av att koncentrationen i blodet inte var tillräckligt hög. Imatinib valdes som hämnmare av transportproteiner som ansvarar för att utsöndra rosuvastatin i gälla. Avsaknaden av en hämmande effekt av imatinib antyder att koncentrationen inne i levercellen inte var tillräckligt hög för att påverka denna process eller att imatinib inte är en tillräckligt potent hämnmare av dessa processer i levern. Gallutsöndring av rosuvastatin visade sig vara mycket potent och koncentrationen i gallan var alltid mycket högre än den i plasma. I människor var exponeringen i gallan jämfört med plasman 617 gånger högre och varierade från 315 till 1198 gånger högre. I grisar var motsvarande värde 1700 gånger högre och varierade från 376 till 11300 gånger högre.

Denna avhandling har visat på betydelsen av gallutsöndring för hur rosuvastatin fördelas i kroppen samt att transportproteiner har stor betydelse för hur mycket som extraheras av levern. Signifikanta skillnader i den systemiska exponeringen observerades framför allt när upptagstransportörer i levern hämmades.
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[Signature]

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