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Drug Transport and Metabolism in Rat and Human Intestine

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Abstract
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One of the aims of this thesis was to investigate the involvement of efflux proteins, such as the P-glycoprotein (Pgp), in the drug transport in different regions of the rat and the human intestine. The intestinal extrusion of intracellularly formed CYP3A4 metabolites, including whether this extrusion might be mediated by Pgp, was also studied. The model drugs used were local anaesthetics (LA), which have been evaluated for inflammatory bowel disease, such as ropivacaine, lidocaine and bupivacaine. The intestinal permeability to LAs was found to be high throughout all intestinal regions of the rat and human intestine. Results from the Ussing chamber model indicated only minor efflux involvement as the drug permeability was higher in the serosa to mucosa transport direction than in the opposite direction. However, the involvement of efflux in the absorption of LAs could not be verified using in situ single-pass perfusion of rat jejunum. The extrusion of the ropivacaine metabolite, 2′,6′-pipecoloxylidide (PPX), was polarized to the mucosal reservoir of the Ussing chamber for both rat and human intestinal samples, and was probably not caused by any Pgp involvement. The expression levels of CYP3A4 and efflux transporters were consistent with the enzymes' activity in human intestine. PPX formation was mediated by CYP3A4 in human intestine, and cyp2c and cyp2d in rat intestine. Species differences were observed, as PPX was formed in rat colon, but not human colon. In conclusion, the permeability of ropivacaine, lidocaine and bupivacaine was not subjected to efflux transport of significance for their intestinal uptake. The transport of ropivacaine metabolites to the mucosal compartment was probably not mediated by Pgp. The Ussing chamber model showed consistent results with those from intestinal microsomes as far as intestinal metabolism is concerned, making it a suitable model for investigations of the interplay of efflux and metabolism.

Keywords: Ussing chamber, microsome, single-pass perfusion, human, rat, intestine, permeability, efflux, P-glycoprotein, metabolism, cytochrome P450, ropivacaine, lidocaine, bupivacaine, PPX

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Till minne av pappa

Papers discussed

This thesis is based on the following papers, which will be referred to by the Roman numerals assigned below in the text:

I. Berggren, S., Hoogstraate, J., Fagerholm, U., and Lennernäs, H.: Characterization of jejunal absorption and apical efflux of ropivacaine, lidocaine and bupivacaine in the rat using *in situ* and *in vitro* absorption models. *Eur J Pharm Sci* 2004, 21, 553-560.

II. Berggren, S., Lennernäs, P., Ekelund, M., Weström, B., Hoogstraate, J., Lennernäs, H.: Regional transport and metabolism of ropivacaine and its CYP3A4 metabolite PPX in human intestine. *J Pharm Pharmacol* 2003, 55, 963-972.

III. Berggren, S., Gall, C., Wollnitz, N., Ekelund, M., Karlbom, U., Hoogstraate, J., Schrenk, D., and Lennernäs H.: Gene and protein expression of P-glycoprotein, MRP1, MRP2 and CYP3A4 in the small and large human intestine.
Accepted for publication in Molecular Pharmaceutics.

IV. Berggren, S., Sohlenius-Sternbeck A-K., Abdel-Rehim, M., Hoogstraate, J., Lennernäs, H.: Regional metabolism and transport of ropivacaine and its metabolites in rat intestine.
In manuscript.

Contents

Abbreviations

ANOVA	analysis of variance
CV	coefficient of variation
CYP	cytochrome P450
EDTA	ethylenediaminetetraacetic acid
F	bioavailability
f_a	fraction absorbed
HPLC	high performance liquid chromatography
KBR	Krebs buffered ringer solution
LA	local anaesthetic (ropivacaine, lidocaine and bupivacaine)
LC-MS	liquid chromatography mass spectrometry
LOQ	limit of quantification
MDR	multidrug resistance
MRP	multidrug resistance-associated protein
M-S	mucosa to serosa
Muc	mucosal compartment
NADPH	nicotinamide adenine dinucleotide phosphate, in the reduced form
P_{app}	apparent permeability
P_{eff}	effective permeability
Pgp	P-glycoprotein
PPX	2´,6´-pipecoloxylidide
rpm	revolutions per minute
RT-PCR	reverse transcription- polymerase chain reaction
SEM	standard error of the mean
Ser	serosal compartment
S-M	serosa to mucosa
TRIS	tris(hydroxymethyl)-aminomethane
UWL	unstirred water layer

Introduction

Oral administration of drugs

A drug can be administered in different ways to obtain either a local or a systemic effect. If a systemic effect is desired, the oral route is often preferred; this is also the most common route for administration. For the patient a solid dosage form, such as a tablet or a capsule for oral use, is convenient as it is easy to handle and to administer in the correct dose. In addition, manufacturers usually have a preference for oral solid dosage forms owing to their low costs of production. However, when a drug is taken orally to obtain a systemic effect, the formulation has to disintegrate, whereupon the drug molecules have to be dissolved in the gastrointestinal fluids, permeate the intestinal membrane, and pass through the major metabolizing organ in the body, the liver, before they can reach their target through the systemic circulation. In drug development it is important to find a drug that, apart from having a specific effect on the target receptor, can overcome all the sequential hindrances from the formulation to the systemic circulation (Rowland & Tozer 1995).

Solubility and permeability

When a drug is taken orally, the tablet/capsule has to disintegrate before the drug can dissolve in the gastrointestinal fluids. Usually, this takes place in the stomach, but when an enteric coating has been used or the drug is given in an extended release formulation, disintegration occurs in the intestine. The formulation excipients chosen are important for the disintegration (Rowland & Tozer 1995; Aulton 2002). Disintegrated particles are dissolved in the gastrointestinal fluids. Adequate levels of drug solubility are required, because the solubility determines the amount of drug available for absorption. The biopharmaceutical classification system (BCS) points to the solubility and permeability as important factors for the intestinal absorption of orally administered drugs (Amidon et al 1995). This classification divides the compounds into four classes: Class I substances defined as having a high permeability and high solubility; Class II ones have a high permeability and low solubility; Class III drugs have a low permeability and a high solubility and class IV is appropriate for drugs with a low permeability and a low solubil-

ity. The limit between having a high and low permeability has been set to a fraction absorbed (fa) of 90%. Thus, if it is to be classified as a highly soluble compound, the highest dose to be given should be soluble in 250 ml of aqueous media in the range from pH 1 to pH 7.5 (Amidon et al 1995; Lennernäs & Abrahamsson 2005).

The intestinal barrier

One prerequisite for an orally administered drug to have a systemic effect is that there is drug uptake from the gastro-intestinal tract. However, the intestine is not only well suited for the absorption of nutrients and drugs, but also it also protects the organism from potentially harmful xenobiotics, which might include drugs. The first hindrance to drug absorption is the acidic environment in the stomach, which can degrade not only bacteria but also some drug substances such as omeprazole (Cederberg et al 1989; Howden 1991). In addition to which, the intestinal membrane along the intestine is covered with a tight epithelium which contains various enzymes that not only degrade nutrients for uptake, but also convert xenobiotics into hydrophilic metabolites to assist in their elimination from the body.

Intestinal drug absorption and bioavailability

Absorption has been defined as the process by which a drug is taken from the site of administration to the site of measurement within the body (Rowland & Tozer 1995). Oral drug absorption is often referred to as drug transfer across the apical membrane of the enterocyte, because the apical membrane is considered to be the rate limiting step for permeation of the membrane (Fagerholm & Lennernäs 1995; Lande et al 1995). Permeability is a general term describing how readily the drug is transferred through a membrane. The specific permeability characteristics of a drug are dependent on its physico-chemical properties, including lipophilicity, charge, size and polar surface area (Rowland & Tozer 1995; Lipinski et al 2001). The rate of absorption is dependent on the permeability, surface area and the concentration gradient over the membrane. The concentration gradient is the driving force for passive diffusion, the most common mechanism for drug membrane transport. Bioavailability (F) is defined as the fraction of the dose that reaches the systemic circulation and can be described as follows:

$$F=fa*(1-Eg)*(1-Eh) \qquad \text{Eq. 1}$$

where fa is the fraction absorbed over the intestinal epithelia, Eg is the gut wall extraction and Eh is the hepatic extraction.

Intestinal pH

The intestinal pH is an important factor for drug permeability as unionized molecules will pass the intestinal barrier most readily (Shore et al 1957). As many drugs have a pKa in the physiological pH range, the permeability might vary with the degree of ionization of the compound according to the Hendelson-Hasselbalch equation (Rowland & Tozer 1995). The lumenal pH in the fasted state of the gastrointestinal tract varies from one intestinal region to another. In the stomach, it has been shown that the pH is approximately 2, in duodenum it is 6.0, from the jejunum to the ileum, it lies in the interval 6.5-7.5 and in colon, it is 7.0 (Evans et al 1988; Dressman et al 1990). Consequently, there could also be a pH gradient across the intestinal membrane as the pH of the blood is constant. This gradient could affect the drug permeability and is, therefore, often applied in *in vitro* permeability screening (Yamashita et al 2000). The microclimate, consisting of an unstirred water layer (UWL) of mucus just adjacent to the membrane, has a slightly lower pH than in the lumen (Hogben et al 1959; Lucas 1983). The UWL might affect the drug permeability especially in *in vitro* models such as the Ussing chamber, but its impact could be minimized with appropriate stirring of the chamber compartment solution (Shiau et al 1985).

Regional intestinal permeability

The upper part of the intestinal tract is the primary site for drug absorption for immediate release formulations. A prerequisite for any extended release formulation is that the permeability of the drug compound is adequate in all parts of the intestine, including the colon, which has a much smaller absorbing surface area (0.25 m^2) compared to the small intestine (120 m^2). When it is intended that an extended release product will release a drug compound from a formulation over, for example, a period of 24 hours, approximately 20 hours of drug release and subsequent uptake may take place in the colon, as the small intestine has a rather constant transit time of 3-4 hours (Kararli 1995).

Metabolism

The liver is the major site of metabolism for orally administered drugs, but it has been shown that intestinal metabolism can also be important for drug bioavailability (Kaminsky & Fasco 1991; Lown et al 1994; Paine et al 1996). The cytochrome P450 (CYP) enzymes mediate phase I metabolism, which includes the oxidation, reduction or hydroxylation of drug compounds. The phase I metabolism might also be followed by phase II metabolism, in the

form of conjugation with for example, glutathione. Metabolism is generally the modification of molecules to make them more water soluble, to facilitate excretion from the body. In humans, it has been shown that CYP3A4 is the most important phase I enzyme involved in drug metabolism as approximately 50% of registered drugs are CYP3A4 substrates (Thummel & Wilkinson 1998). The CYP3A subfamily constitute 20% of CYP content in liver and 50-70% of CYP content in the intestine (Watkins et al 1987; Paine et al 1997).

Metabolism is often measured using liver microsomes, but these results cannot be extrapolated to determine the intestinal metabolism as there might be a lack of correlation between hepatic and intestinal cyp isoforms (Aiba et al 2003). For example cyp3a1 and cyp3a2 are expressed in rat liver but cannot be detected in rat intestine (Zhang et al 1997; Matsubara et al 2004) where instead, the isoform cyp3a9 has been detected (de Waziers et al 1990; Takara et al 2003). Cyp2d has been found to be present in rat intestine to a small extent (Aiba et al 2003; Lindell et al 2003). There are conflicting results on cyp2c11 or cyp2c6 expression in rat intestine, as one study found that cyp2c was expressed in rat intestine at approximately the same level as in the rat liver, at least at the mRNA level (Lindell et al 2003). In other studies using Western blot cyp2c was not found at all (Johnson et al 2000).

Drug transporters

P-glycoprotein

In 1976 Juliano and Ling found a correlation between the degree of drug resistance of Chinese hamster ovary cells and the presence of a 170 kDa membrane glycoprotein. The glycoprotein appeared to be unique to mutant cells displaying altered drug permeability, so they named it P-glycoprotein where the ”P” stands for “permeability” (Juliano & Ling 1976). Pgp is a phosphoglycoprotein belonging to the ATP (adenosine triphosphate)-binding cassette (ABC) transporter super-family. ABC transporters have a highly conserved ATP binding region, which is characteristic for these transporters. Over 200 ABC transporters are known and they exist in a wide variety of species, ranging from bacteria to humans, and are found in association with importation or export nutrients, amino acids, sugars, peptides or hydrophobic substances (Higgins 1992; Leveille-Webster & Arias 1995). The P-glycoprotein, (Pgp/ABCB1), can transport compounds with a broad range of chemical structure out of a cell through the consumption of energy. This phenomenon has been referred to as multidrug resistance (mdr)(Hunter & Hirst 1997), a name which has been given to the genes encoding for Pgp. Humans have one gene for multidrug resistance, the MDR1. Mouse and rodents have two genes for multidrug resistance, mdr1 and mdr3, these are also referred to as mdr1b and mdr1a, respectively. In mice these genes have

92% amino acid identity and they probably fulfill the same functions as human MDR1. Mdr1a is the predominant isoform present at the apical surface of the intestine (Brady et al 2002). Neither human MDR2, nor mouse mdr2 are involved in multidrug resistance, but rather they play a role in the transport of phospholipids into bile (Smit et al 1993).

One determinant for interaction with Pgp is the relative hydrophobicity of the interacting molecule. Substrates for Pgp have a partition coefficient (octanol/water) of approximately +1 or greater (Hunter & Hirst 1997). Pgp transports large hydrophobic, uncharged or slightly positively charged molecules, although it seems as if some hydrophilicity is needed (Sarkadi & Muller 1997; Stein 1997).

It was discovered that Pgp was expressed not only in tumor cells but in many normal cells in various organs such as the liver, kidney, intestine and brain in humans and animals (Fojo et al 1987; Thiebaut et al 1987; Cordon-Cardo et al 1990). It has been suggested that the function of Pgp is to protect the body and important organs, such as the brain, from naturally occurring toxic substances. The contribution of Pgp in the blood-brain barrier (BBB) has been shown using knockout mice lacking the mdr gene, and drugs such as ivermectin and loperamide exhibited a much higher degree of brain toxicity when Pgp was absent (Schinkel et al 1994; Schinkel et al 1996). The function of Pgp in the intestine in not fully understood but it has been suggested that intestinal efflux transport by Pgp and other efflux proteins can affect the rate and extent of drug absorption and metabolism in rat and human intestine (Gan et al 1996; Terao et al 1996; Lown et al 1997; Kruijtzer et al 2002). Intestinal transporters, such as Pgp, could also cause drug-drug interactions due to changes in drug pharmacokinetics (Lin & Yamazaki 2003). It could also be possible to increase the bioavailability after oral administration and transport across the blood-brain barrier for some drugs and to further understand drug-drug interactions.

Multidrug resistance-associated protein, MRP

In 1992, Cole and colleagues found the first non-Pgp efflux protein in multidrug resistant cells (Cole et al 1992). The multidrug resistance-associated protein, MRP, is a 190 kDa transport protein belonging to the family of ABC transporters and, like Pgp, it gives rise to drug resistance in tumor cells (Lautier et al 1996). MRP has overlapping substrate specificities with Pgp and is expressed in almost the same tissues as Pgp, with the exceptions of the liver and brain (Evers et al 1996; Sugawara et al 1997). MRP transports some negatively charged compounds and, particularly conjugates of lipophilic substances with gluthatione, glucuronate or sulphate (Sarkadi & Muller 1997).

The MRP family consists of at least six homologous members, MRP1-6. Of these, it has been reported that MRP1, MRP2 (cMOAT) and MRP5 are

expressed in the small and/or the large intestine in humans and rats. *In vitro* experiments have revealed that MRP1, MRP3, and MRP5 seem to be expressed at the basolateral membrane of enterocytes, mediating transport in the absorptive direction. In contrast, MRP2 is expressed at the apical membrane, transporting its substrates to be secreted at the apical side of the enterocyte, thus it may be involved in the efflux of drugs into the intestinal lumen (Kool et al 1997; Borst et al 1999).

The apical recycling theory

CYP3A4 and Pgp have almost complete overlap in substrate specificity and they are commonly localized near the apical membrane of the enterocyte (Wacher et al 1995; Watkins 1997; Cummins et al 2001). They are furthermore both regulated by the orphan nuclear pregnane X receptor (PXR)(Kliewer et al 1998; Lehmann et al 1998; Synold et al 2001). It has been suggested, in the apical recycling hypothesis, that CYP3A4 and the Pgp cooperate in the transport and metabolism of drugs in the intestinal membrane. According to this theory, Pgp slows down the drug presentation rate and increases the drug exposure to the CYP3A4 enzyme by recycling the drug in the enterocyte (Benet et al 1996; Watkins 1997; Ito et al 1999; Johnson et al 2001a). It has also been suggested that this interplay includes Pgp-mediated active transport of the metabolites formed to the intestinal lumen (Figure 1)(Gan et al 1996; Hochman et al 2001).

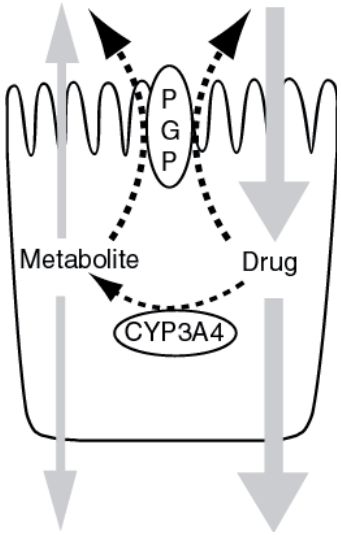

Figure 1. Schematic illustration of how CYP3A4 and the P-glycoprotein (Pgp) might cooperate in the enterocyte. Pgp could slow down the drug presentation rate to the enzyme by apical recycling. In addition Pgp might aid the apically directed transport of the metabolite formed.

Methods for studying drug absorption and metabolism

The first step in predicting the permeability and solubility properties of a drug includes the determination of the partition of the drug between water and octanol (i.e. logD and logP), as there is a correlation of permeability to the water solubility properties of the drug. Estimation of the polar surface area (PSA) of the drug molecule could also be used for prediction of the drug membrane permeability (Palm et al 1997). Although these methods could be used for a rough prediction of the passive permeability, more complex methods are generally required to evaluate the intestinal absorption process of a specific candidate drug. There are many methods to choose from, all of which have their pros and cons, and the method chosen will depend on the purpose of the study (Barthe et al 1999).

Cell lines

The use of cultured cells, which form polarized monolayers, to determine the intestinal permeability characteristics of drugs has expanded in recent years. The most commonly used model for drug permeability is the Caco-2 cell model (human colon adenocarcinoma cells). Caco-2 resemble cells in the small intestine, despite its colonic origin, and this model can be used to predict the intestinal permeability in humans (Hilgers et al 1990; Artursson & Karlsson 1991). The cells can be utilized for low throughput mechanistic studies, as well as high-throughput screening using automated systems, to render the screening of drugs for their permeability properties more effective (Garberg et al 1999; Tannergren et al 2001).

The function of Pgp has been extensively studied using the Caco-2 model, as Pgp is expressed on the apical membrane of Caco-2 cells (Hunter et al 1993). A higher permeability in the basolateral to apical direction (b-a) than the to apical to basolateral (a-b) has generally been considered as a requirement to identify active transport, such as Pgp efflux in the apical membrane, efflux is often defined as a ratio of b-a / a-b that is more than two (Polli et al 2001). However, the expression levels of efflux transporters in cultured cells does not always reflect the expression of transporters in the human intestine (Sun et al 2002). It has been reported that Pgp expression is dependent on culturing conditions, such as the number of days in culture and the passage number, which makes the interpretation more difficult (Hosoya et al 1996; Anderle et al 1998). Apart from Caco-2 there are other cell lines, derived from pig kidney (LLC-PK1) and dog kidney (MDCK), which are often used to study drug efflux. However, these often need to be transfected with transporter genes to express significant levels of the protein (Schinkel et al 1995; Evers et al 1996; Soares-Da-Silva & Serrao 2000).

As far as the CYP3A4 metabolism is concerned, the Caco-2 cell model is not representative as the CYP3A4 is not expressed efficiently. The gut wall

extraction may be underestimated, and consequently the intestinal permeability of drugs metabolized by CYP3A4 would be overestimated. This has given rise to the development of CYP3A4 induced cell lines that better reflect CYP3A4 activity in the human intestine (Schmiedlin-Ren et al 1997; Engman et al 2003). There is a major drawback to the cell culture models when used for the kind of studies included in this thesis arising from the inappropriate levels of intestinal enzymes in relation to those of the efflux transporters. Thus, expression levels of enzymes and transporters fail to correspond to the levels *in vivo*. As a result of this, interpreting the results can be problematic as far as the interplay of enzymes and transporters in the intestine is concerned.

The Ussing chamber

The Ussing chamber was named after its inventor, the Danish professor, Hans Ussing, and the first application of the new technique was for studies of ion transport in frog skin (Ussing & Zerahn 1951). Later, the Ussing chamber was utilized for the measurements of drug tissue permeability to predict intestinal drug absorption (Grass & Sweetana 1988; Ungell et al 1998; Gotoh et al 2005). Over the years, the original glass chambers have gradually been replaced by other materials, such as different types of plastics, nowadays Ussing chambers come in all different sizes to suit specific applications. The Ussing chamber is a unique method, with several advantages over other *in vitro* methods for applications in intestinal drug transport and metabolism research (Madden et al 1989; Tjia et al 1991; Makhey et al 1998). When evaluating efflux in the Ussing chamber, the ratio of the permeability from the serosal to mucosal chamber half (S-M) is compared with the permeability in the reverse direction (M-S), corresponding to the a-b / b-a ratio used for studies with cultured cells as mentioned in the previous section. The most important advantage of the Ussing chamber compared to cell culture models is that it enables the use of excised tissues, which ensures that the appropriate membrane characteristics for drug transport are investigated. As the transporters and enzymes are expressed at physiologically relevant levels, it is possible to study the combined effect of both enzymes and active transporters in the intestine (Rogers et al 1987; Smith et al 1988). The Ussing chamber is also well suited to study the differences of drug transport in different parts of the intestine, which is more difficult with cultured cells (Ungell et al 1998; Naruhashi et al 2001). It is also possible to continuously measure the tissue viability by means of its ability to actively transport ions; viability measurements usually include measuring the resistance, short circuit current and potential difference.

Tissues used for Ussing chamber investigations are most often taken from common laboratory animals such as the rat which makes the model rather simple to use. However, using animal tissues give rise to problems in the

interpretation of permeability data owing to species differences, especially as there could be differences in the expression levels of enzymes and transporters and also physiological differences. The desired material for drug permeability studies would be human intestinal samples, but generally the access to such material is sparse, at least in the amounts required for the Ussing chamber. Because of the difficulties in obtaining human intestinal tissue, there are methods under development to be able to use biopsies, which would make it possible to use volunteers instead of tissues from intestinal cancer surgery (Wallon et al 2005).

Intestinal perfusion

Intestinal perfusion makes it possible to investigate the absorption of drugs in different parts of the intestine with the blood flow and other physiological characteristics being maintained. In animals such as the rat, the *in situ* single-pass perfusion is commonly applied for drug permeability studies. The intestinal segment to be studied is cannulated using a surgical procedure and perfused with the drug in solution. The effective permeability can be calculated using the parallel tube model (Komiya et al 1980). In humans, the Loc-I-Gut® technique (Knutson et al 1989) is used to inflate two balloons to isolate one part of the intestine for which the effective permeability can be calculated using the well-mixed model (Lennernäs et al 1992). Both the single-pass perfusion and the Loc-I-Gut® methods can be used to estimate the permeability of passively transported drugs and can also be used to investigate the *in vivo* impact of carrier-mediated drug transport.

Microsomes

Liver microsomes are probably the most common way of studying metabolism *in vitro*. The experiments are relatively easy to perform and often give a great deal of information on the metabolism of drugs. The methods used for isolating the microsomal fraction of liver cells, which usually include homogenization of tissue and then several centrifugation steps, have also been applied to intestinal cells. Microsomes contain cytochrome P450 enzymes and can be used to determine which enzymes are involved in drug metabolism. This enables enzyme kinetic variables to be calculated. Microsomes can also be used to detect drug-drug interactions caused by metabolism (Kaminsky & Fasco 1991).

Ropivacaine, lidocaine, bupivacaine and inflammatory bowel disease

Lidocaine was the first local anaesthetic (LA) to be tested as a local treatment of proctitis and ulcerative colitis. The mechanism behind its action is not fully understood, but it is known to be effective in restoring inflamed intestinal mucosa (Björck et al 1989; Lang & Peppercorn 1999; Björck et al 2002). The anti-inflammatory effects of LAs have been reviewed by Hollmann (Hollmann & Durieux 2000). Ropivacaine was a new structural analogue to lidocaine, with a longer duration of action and with more extensive preclinical documentation available, including investigations of CNS toxicity, which was a major concern as a potential side-effect. At about the same time as onset the of the work for this thesis, ropivacaine was evaluated as a local treatment for ulcerative colitis in clinical trials with promising results (Arlander et al 1996; Martinsson et al 1997; Björck et al 2002). A tablet for colonic delivery has also been formulated using ropivacaine as a model drug (Ahrabi et al 2000).

The application of LAs as a local intestinal treatment initiated the usage of ropivacaine, lidocaine and bupivacaine as model drugs (Figures 2 and 3) in studying the intestinal uptake and the efflux in human and rat intestine. There have also been some conflicting results whether inflammatory bowel disease (IBD) has a correlation to the expression of the mdr1 gene (Annese et al 2006). The CYP3A4 ropivacaine metabolite 2′,6′-pipecoloxylidide (PPX) has been shown to be an active metabolite with analgesic effect 12 times lower than that of ropivacaine (Arlander 2003; Ekatodramis et al 2003). PPX has been shown to be of minor importance owing to extensive CYP1A2 metabolism in the liver (Arlander et al 1998), but, for example, when administered via epidural injection, PPX levels increase rapidly because the half-life is longer than that of ropivacaine and because of the lack of CYP1A2 involvement. In the intestine CYP1A only has a minor influence of and it is expected that PPX will be the major metabolite. Ropivacaine and PPX were, therefore, chosen as the model compounds for studying the transport and metabolism in the intestine of rat and humans, and to investigate the involvement of carrier-mediated efflux (Figure 3).

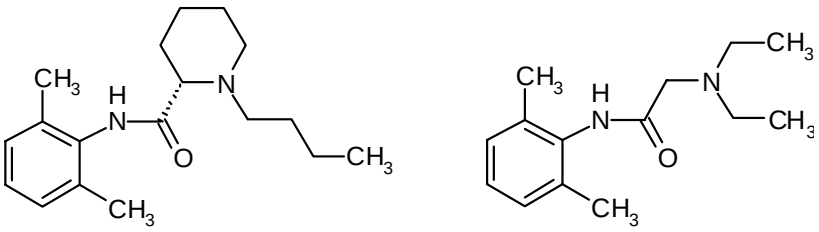

Figure 2. The chemical structures of bupivacaine (left) and lidocaine (right).

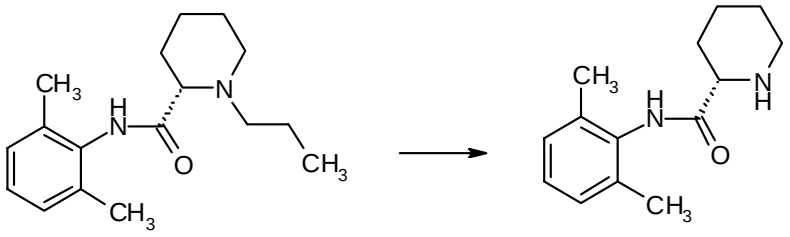

Figure 3. The chemical structures of ropivacaine and its CYP3A4 metabolite PPX

Aims of the thesis

The main objective was to investigate the involvement of efflux transporters, such as the P-glycoprotein (Pgp), in the intestinal permeability of drugs and in the transport of metabolites in different regions of rat and human intestine. The regional perspective of efflux and metabolism was also evaluated to determine the expression levels of efflux transporters and CYP3A4 in human intestine.

The specific aims were:

- to compare the permeability and efflux transport of ropivacaine, lidocaine and bupivacaine using different absorption models, such as the *in situ* single-pass perfusion and excised tissues in Ussing chambers for rat jejunum (Paper I).

- to examine the mechanism behind ropivacaine permeability and the extrusion of its metabolite PPX in the rat and human jejunum, ileum and colon using the Ussing chamber technique (Papers II and IV).

- to characterize the intestinal expression levels of Pgp, MRP1, MRP2 and CYP3A4 in excised tissue from the jejunum, ileum and colon of the human intestine at both mRNA and protein level, using both RT-PCR and Western blot analysis, respectively (Paper III).

- to investigate the enzyme kinetics of ropivacaine metabolism in rat intestinal microsomes from different intestinal regions and to identify the enzymes involved in ropivacaine metabolism in the rat intestine (Paper IV).

Materials and methods

Ussing chamber - experimental procedure

Human intestine (Paper II)

Human intestinal segments were obtained from patients undergoing gastric bypass or surgery for either pancreatic cancer (jejunum) or intestinal cancer (ileum and colon). None of the patients included in the intestinal Ussing chamber studies had undergone treatment with cytotoxic substances or radiation therapy prior to their surgery as such treatment is known to affect the intestinal characteristics and permeability (Nejdfors et al 2000). The excised specimens were inspected visually to ensure that they had normal morphology without any signs of inflammation or cancer infiltration. The intestinal segments were transported to the laboratory immediately after resection in room temperature Krebs buffered Ringer (KBR) solution with a pH of 7.4 that had been saturated with carbogen (95% O_2 and 5% CO_2). Each mucosa was carefully dissected from the serosa-muscle layer and mounted in the Ussing diffusion chambers with 1.78 cm^2 of exposed tissue area (Navicyte, San Diego, CA, USA). The reservoirs of the Ussing diffusion chambers were filled with 6 mL modified Krebs Buffer (KBR)(pH 7.4, 290 mOsm kg^{-1}) containing (mM) NaCl 108, KCl 4.7, Na_2HPO_4 1.8, KH_2PO_4 0.6, $NaHCO_3$ 16, $MgSO_4$ 1.2, Na-pyruvat 4.9, Fumarate 5.4, L-glutamat 4.9, D-glucose 11.5 and $CaCl_2$ 1.25. Carbogen was continuously bubbled through the KBR buffer. The buffer was circulated by gas lift at a temperature of 37°C (Figure 4). The transepithelial potential difference (PD) was measured just before the start of the experiment to check the viability of the resected segment, using a Voltmeter (Millicell-ERS Millipore, Sweden) equipped with a pair of Ag/AgCl electrodes embedded in 3M KCl agar. The experiments were started (t=0) within 60 min of dividing the blood vessels during the surgical procedure, by replacing the buffer in the reservoirs with test solutions. The donor solution contained viability and integrity markers, ^{3}H-D-glucose and ^{14}C-mannitol. Compounds used as inhibitors were added on both sides of the segment. Samples of 1 mL were withdrawn from both reservoirs every 30 min and the volume of each sample was immediately replaced by the relevant starting solution. The experiment was continued for 150 min. Samples were immediately frozen, at -20°C, pending analysis. The study was approved by the Ethics Committee of Lund University.

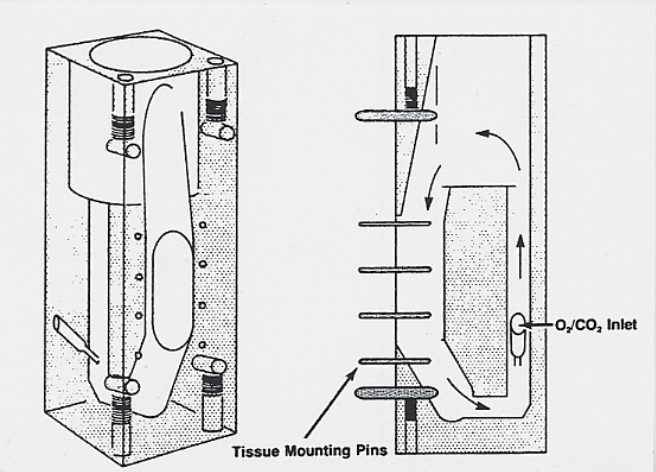

Figure 4. Illustration of the Ussing chamber system, tissue is mounted on the mounting pins before the two halves of the chamber are connected. Each reservoir/compartment is filled with a KBR solution circulated by gas lift at the O_2/CO_2 inlet (Grass & Sweetana 1988).

Rat intestine (Papers I and IV)

Male Sprague-Dawley rats were received from B & K Universal AB, Sollentuna, at least one week prior to the experiment. They were kept under a 12 hour light/dark cycle with free access to pellet food (R70, Lactamin AB, Vadstena, Sweden) and tap water. Anaesthesia was introduced with Forene® (isofluran) (Abbot Scandinavia AB, Kista, Sweden) and the intestinal segments were excised, washed and immediately put in cold oxygenated (95% O_2 and 5% CO_2) Krebs Buffered Ringers solution (KBR)(Ungell et al 1992) to be stabilized for about 30 minutes. Segments from the jejunum was collected approximately 10 to 35 cm distally from the ligament of Treitz, ileum was collected 0 to 30 cm proximal to the caecum and colon 5 to 10 cm distally from the caecum. Segments identified as being free from Payer's patches (by visual inspection) were cut along the mesenteric border, and the serosa layer was removed. The residue was mounted in the Ussing chambers (Plexiglas chambers, AstraZeneca). The compartments were filled with 10 mL KBR (37°C, pH 7.4) continuously bubbled with 5% CO_2 in O_2 (50±10 mL/min) and were stirred with paddles at 225 rpm. The experiments were started by replacing the KBR with the test solutions. The pH was 7.4 in the serosal compartment and either 7.4 or 6.5 in the mucosal compartment. The temperature was kept at 37°C throughout the experiment using heat blocks. In Paper I, three donor samples of 100 µL were taken as follows,

immediately after the start of experiment, after 120 min and at the end of the experiment, at 180 min. In Paper IV samples of 500 µL were withdrawn from both the receiver and donor compartments every 30 minutes and the volume was immediately replaced with the relevant test solution for the chamber compartment, at 37°C. The viability of the intestinal segments was monitored by recording the electrical parameters as follows: PD (potential difference), SCC (short circuit current) and R (resistance) throughout the experiment as has been described previously (Polentarutti et al 1999). Segments having an initial PD of less than 4 mV were not included in the study. The study was approved by Stockholm Södra Animal Research Ethics Board.

In situ single-pass intestinal perfusion (Paper I)

Male Sprague-Dawley rats from Charles River AB, Uppsala, were housed under controlled conditions (22.5°C, 50% air humidity and 12 hours light-dark cycle) prior to the experiment. They had free access to tap water and pellet food (R36, Lactamin AB, Vadstena, Sweden) until 14-20 hours prior to the experiment. Anaesthesia was introduced by an intraperitoneal injection of 150 mg/kg of Inactin®-Byk (thiobutabarbital sodium). The animals were then put on a heating pad (CMA-150, Carnegie Medicine AB, Stockholm, Sweden) to maintain a body temperature of 37°C. To facilitate normal breathing a polyethylene tube was introduced into the trachea. The abdominal cavity was opened by a midline incision of approximately 5 cm. The jejunum was located and an approximately 10 cm long segment was cannulated on each side with non-PVC plastic tubing of 4 mm outer diameter (Codan Triplus AB, Kungsbacka, Sweden). A loop of about 15 cm of the plastic tubing was inserted into the abdominal cavity to preheat the perfusate before entering the segment. The jejunal segment was rinsed with about 20-30 mL of saline until the outlet perfusate was clear. The tubing was connected to a syringe with perfusion solution which was pumped through the segment at a flow rate of 0.2 mL/min by an infusion pump (Model 22, Harward Apparatus Company, USA). After an equilibration period of 45 min, a fraction of perfusate was collected every 15 min. The total perfusion time was 105 min. Samples were frozen immediately and stored at -20°C pending analysis.

The perfusion solution used was a 70 mM phosphate buffer at pH 6.5 containing 5.4 mM potassium chloride, 48 mM sodium chloride, 35 mM mannitol, 10 mM D-glucose and 1 g/L PEG 4000 (polyethylene glycol 4000). ^{14}C- PEG 4000 (2.5 µCi/L) and ^{3}H-D-glucose (10 µCi/L) were included as markers for water flux and active transport. Antipyrine was added as a marker for passive transport. Osmolality was approximately 290 mOsm kg^{-1}.

Adsorption and stability of the drugs

The potential adsorption of ropivacaine, lidocaine, bupivacaine and PPX to the perfusion catheters, the Ussing chambers and test tubes was investigated prior to the experiments. There was no evidence of precipitation, chemical instability or of adsorption to the materials used. The stability of antipyrine has been demonstrated previously (Lennernäs et al 1994).

Intestinal microsome method (Paper IV)

Preparation of microsomes

The intestines from male Sprague-Dawley rats were excised and regional parts (of the jejunum, ileum and colon) were collected as previously described for the Ussing chamber method. Samples from the duodenum were collected 0-10 cm distally from the ligament of Treitz. Immediately upon resection the segments were rinsed in ice-cold 0.15 M KCl. Subsequently, the intestinal segments were opened longitudinally and the mucosa was gently scraped off the inside of the intestine with a metal slide. The mucosal samples were frozen in liquid nitrogen and stored at -70°C.

The mucosal samples from 3-4 rats were thawed on ice, pooled, and the intestinal microsomes were prepared. The homogenization was carried out with a Potter-Elvehjelm homogenizer in ice-cold 0.01 M phosphate buffer, with pH 7.4, comprised of 1.15% KCl and 40 μg phenylmethylsulphonyl fluoride/mL by using ten strokes up and down at 1140 rpm. The samples were centrifuged at 9750 g for 20 min. The supernatant containing cytosol and microsomes (S9-fraction) was centrifuged at 105000 g for 70 minutes. The microsomal pellet was washed in 1.15% KCl and resuspended in 0.05 M phosphate buffer, pH 7.4. Aliquots of the microsomes were stored at –70 °C. The microsomal protein concentration was determined using the method introduced by Lowry (Lowry et al 1951). A protein assay kit (Bio-Rad Laboratories, Richmond, CA, USA) with bovine serum albumin as the standard was used.

Incubations

The incubation mixture, of which there was 200 μL, contained microsomes (at a concentration of 2 mg/mL), ropivacaine and NADPH in 0.05 M phosphate buffer, pH 7.4. Ropivacaine was dissolved in methanol, giving a concentration of 1% in the incubation mixture. Under the conditions used, the formation of metabolites increased linearly with protein concentration and time and less than 10% of the substrate was metabolised. The microsomes were preincubated with NADPH for 4 minutes at 37 °C. The reaction was

started with the addition of ropivacaine and was terminated after 18 minutes by addition of 200 μL of acetonitrile. The samples were centrifuged and the supernatant was evaporated to dryness under a stream of nitrogen. The samples were reconstituted in 120 μL water, and 20 μL of internal standard was added before being injected on to an LC-MS-MS (Micromass Z-spray QII). When inhibitors were used, they were first dissolved in acetone, giving an concentration of 1% in the incubation mixture, whereupon they were preincubated for 15 minutes prior to the start of the experiment.

Western blot and RT-PCR procedure (Paper III)

Intestinal tissues

Intestinal tissues were obtained from patients as described previously when the procedure adopted for the Ussing chamber was discussed for human intestine. The segments did not show any signs of inflammation or cancer infiltration. Immediately after resection, the intestinal segments were snap frozen using liquid nitrogen and stored at -80°C. Prior to surgery, the patients were informed about the investigation and they gave their informed consent to participate in it. The study was approved by the Ethics Committees of Lund University and Uppsala University.

Western blot

The frozen tissue samples were cut into pieces on dry ice and were homogenized in 0.1 M Tris-HCl buffer (pH 7.6) supplemented with EDTA and an EDTA-free protease inhibitor cocktail (Complete® tablets, Roche, Mannheim). The homogenate was centrifuged at 8500 g (4°C) for 15 min and the supernatant was centrifuged at 100000 g (4°C) for 30 min. The pellet was dried and resuspended in the same buffer as used for homogenization. Aliquots were snap frozen with liquid nitrogen and stored at –80°C. Membrane proteins (25 μg) were separated with SDS-PAGE and electro-blotted on Immobilon^{TM}-P membranes (Millipore, Neu-Isenburg, Germany). The blots were blocked with 10% (w/v) non-fat milk in Tris-buffered saline (TBS)/Tween (0.05% (v/v)) for 1 hour and subsequently probed with the primary antibody in TBS/Tween for 1 hour. The immunoreaction was visualized using a horseradish peroxidase-conjugated goat anti-mouse antibody diluted in TBS/Tween, followed by chemiluminescence (ECL-plus technique, Amersham, Braunschweig, Germany).

Antibodies

For Pgp monoclonal antibody C494, 0.1 mg/mL (Alexis Biochemicals, Grünberg, Germany) was diluted in the ratio 1:1000. As secondary antibody Immunopure® goat anti-mouse IgG (Pierce, Rockford, USA) was diluted in the ratio 1:25000. CYP3A4 murine monoclonal antibody WB-MAB-3A1°Ab (Gentest Corp. BD Biosciences, Woburn, USA) was diluted in the ratio 1:500. This antibody also reacts with CYP3A5 and CYP3A7, enzymes which, it has been reported, are not expressed in the human intestine to a noteworthy extent (Zhang et al 1999; Koch et al 2002). As CYP3A4 secondary antibody anti-mouse IgG HRP2°Ab (Gentest Corp. BD Biosciences, Woburn, USA) was diluted in the ratio 1:20000. For MRP1 and MRP2, the monoclonal antibodies MRPm6 and M_2III-6 (Alexis Biochemicals, Grünberg, Germany), respectively, were diluted in the ratio 1:100. The same secondary antibody as for Pgp was used.

RT-PCR

Approximately 0.5 g of frozen tissue sample was homogenized in 2 mL ice-cold guandinium thiocyanate solution containing 1% mercaptoethanol. Homogenates were centrifuged at 2200 g (4°C) for 10 min. The supernatant of the homogenate, 1.6 mL, was put on top of 2.4 mL cesium chloride solution in pre-cooled vials which were centrifuged at 100000 g (20°C) for 20 hours. The pellet was washed with 400 µL of ice-cold 70% ethanol, and was centrifuged at 17500 g (4°C) for 5 min. The pellet was resuspended in 20 µL diethylpyrocarbonate-H_2O and was stored at –80°C. Reverse transcription polymerase chain reaction (RT-PCR) was conducted according to the method described by Conrad et al (Conrad et al 2001).

Chemical analysis

HPLC (Papers I and II)

Ropivacaine, lidocaine, bupivacaine and PPX were assayed using reversed-phase HPLC. A sample of volume 60 µL was injected on the column (µ-Bondapak C18 9 × 150 mm, Waters) at a flow rate of 1.0 mL/min. The mobile phase consisted of phosphate buffer (pH 8.0, I=0.05) with 60% v/v acetonitrile, a UV detection wavelength of 220 nm was used. Ropivacaine, lidocaine and bupivacaine were eluted between 4 and 6 minutes. For the quantification of PPX, a volume of 120 µL was injected on the column (Symmetry C8 3.9 × 150 mm) with guard column (Symmetry C8 3.9 × 20 mm, Waters) at a flow rate of 1.0 mL/min. The mobile phase consisted of phos-

phate buffer (pH 2.0, I=0.05) with 23% v/v acetonitrile and 5 mM 1-octane sulfonic acid, UV detection wavelength 210 nm. PPX was eluted at 9.5 min.

Liquid scintillation method (Papers I and II)

The concentrations (dpm/mL) of ^{3}H-labelled D-glucose and ^{14}C-labelled mannitol were measured using liquid scintillation counting (Tricarb 1900 CA, Packard Instruments) after addition of 8 mL scintillation liquid (Ready Safe, Beckman Fullerton, CA, USA).

LC-MS method (Paper II)

Analysis of metabolites, other than PPX, related to ropivacaine was performed using HPLC with electrospray ionisation mass spectrometry. The HPLC system consisted of a binary pump with an autosampler (respectively, HP 110 G1312A and G1313A, both supplied by Hewlett-Packard). Separation was achieved on a reversed phase column (Symmetry C8 1.0 × 150 mm, Waters) with guard column (Opti-Guard 1.0 × 10 mm, Alltech Associates, Deerfield, IL). The mass spectrometer was a Quattro II (Micromass Ltd, Manchester, UK) with an electrospray ion source and cross-flow counter-electrode, cone voltage 30 V and 60 V in alternate scans. A gradient program was used and the mobile phases consisted of A: 0.03% (v/v) trifluoroacetic acid in deionised water and B: 0.03% trifluoroacetic acid in water:acetonitrile (40:60 v/v) with the content of phase B increasing by 2% every minute. The flow rate was 0.04 mL/min.

LC-MS method (Paper IV)

The HPLC apparatus included two pumps (Shimadzu LC10Advp, Shimadzu Corporation, Kyoto, Japan) and an autosampler, CTC-Pal (CTC Analytics AG, Zwingen, Switzerland). The columns used were YMC basic, 100 × 2.1 mm, 3 μm (YMC Europe GMBH, Schermbeck, Germany) and optiguard (C8 1 × 10 mm) (Optimize Technologies Inc., Oregon, USA). A VICI, Valco C4W valve (VICI Valco Instrument Inc., Houston, USA) was used between analytical column and mass spectrometer. Gradient HPLC was used with mobile phases A and B containing acetonitrile and water, (10+90) and (80+20), respectively, with 0.1% formic acid. The flow rate was 0.15 ml/min. All experiments were conducted using a triple quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with a Z-electrospray interface (ESI) operated in positive ion mode. The source block and desolvation temperatures were 150^{o}C and 250^{o}C, respectively. Nitrogen was used as both the drying and nebulizing gas, while argon was used as the collision gas. The capillary voltage was 3.2 kV and sampling cone voltage was 38-40 V. The scan mode was “multiple reaction monitoring” using the precursor

ion at m/z (M+1) (m/z: 291, 291, 282, 275, 233) and after collisional dissociation, the product ions 126, 126, 133, 126 and 84 were used for the analysis of 3-OH-ropivacaine, 4-OH-ropivacaine, IS, ropivacaine and PPX respectively. The validation showed that the method is selective, accurate and sensitive. The quality of the determination for ropivacaine and its metabolites was satisfactory and within the quality control samples (QC) acceptance criteria of $\pm$ 15% of nominal value. The limit of quantification (LOQ) was 4.9 nmol/L.

Data analysis

Permeability calculations

Ussing chamber experiments

The apparent permeability, P_{app}, for the Ussing chamber experiments including ropivacaine, lidocaine, bupivacine, PPX, ^{3}H-D-glucose and ^{14}C-mannitol was calculated using Eq. 2 (Papers I, II, IV), whereas the extrusion rate of metabolite to mucosal or serosal compartments (Papers II and IV), was calculated according to Eq. 3. The permeability efflux ratio was calculated from Eq. 4.

$$P_{app} = \frac{dQ}{dt} \cdot \frac{1}{A \cdot C_0} \qquad \text{Eq. 2}$$

$$\text{Extrusion rate} = \frac{dQ}{dt} \cdot \frac{1}{A} \qquad \text{Eq. 3}$$

dQ/dt is the steady-state appearance rate, A is the exposed tissue area, and C_0 is the donor concentration.

$$\text{Permeability efflux ratio} = \frac{P_{app\ S\text{-}M}}{P_{app\ M\text{-}S}} \qquad \text{Eq. 4}$$

P_{app} is the apparent permeability calculated according to Eq. 2 and S-M and M-S denotes the transport direction; serosal to mucosal compartment (S-M) or mucosal to serosal compartment (M-S).

Single-pass perfusion experiments

Calculations of the absorption parameters from single-pass perfusions (Paper I) were obtained from concentrations of the outlet perfusate under steady-state conditions, i.e. when the outlet concentration of ^{14}C-PEG 4000 was stable over time. The net water flux (NWF) was calculated according to Eq. 5.

$$NWF = \frac{(1 - PEG_{out}/PEG_{in}) \cdot Q_{in}}{L} \qquad \text{Eq. 5}$$

where PEG_{in} and PEG_{out} were the inlet and outlet concentrations of ^{14}C-PEG 4000, respectively. Q_{in} was the inlet flow rate (0.2 mL/min). L was the length of the segment, measured after 45 minutes of perfusion. The effective permeability (P_{eff}) was calculated according to the parallel tube model (Komiya et al 1980) as shown in Eq. 6.

$$P_{eff} = \frac{Q_{in} \cdot \ln(C_{in}/C_{out})}{2\pi rL} \qquad \text{Eq. 6}$$

where Q_{in} is the flow rate (0.2 mL/min), and C_{in} and C_{out} were the inlet and outlet concentrations, respectively. $2\pi rL$ is the mass transfer surface area within the jejunal segment with an assumed radius of 0.18 cm.

Statistical analysis

In Papers I, II and IV, the statistical analysis was made using a one way analysis of variance, ANOVA, and the identification of the significances was carried out using post hoc tests, Scheffes (Papers I and II) and Tukeys (Paper IV). P-values of less than 0.05 were considered to be statistically significant. Values are expressed as the mean±SD (Paper I) or the mean±SEM (paper II and IV). In Paper III the statistics were calculated using the Wilcoxon rank sum and the Kruskal-Wallis one way analysis of variance. The relationship between mRNA and protein was made using a correlation analysis of the plots. In Paper IV the significance level used was set at a p-value of less than 0.01 owing to the number of variables studied.

Results and discussion

Intestinal permeability of LAs

The permeability of three local anaesthetic (LA) compounds, ropivacaine, lidocaine and bupivacaine were investigated in rat jejunum using an *in situ* single-pass perfusion model (P_{eff}) and *in vitro* excised jejunal segments in the Ussing chamber (P_{app}) (Paper I). The Ussing chamber method was also used to determine the intestinal permeability of ropivacaine and its N-dealkylated CYP3A4 metabolite PPX in the jejunum, ileum and colon of rat and human excised intestinal tissue (Papers II and IV). Permeability coefficients of LAs and PPX corresponded to a complete intestinal absorption, i.e., a fraction dose absorbed of > 90% (Lennernäs 1997; Ungell et al 1998) and the compounds could, tentatively, be classified as having a high permeability according to the BCS (Amidon et al 1995). The permeability of ropivacaine in the absorptive direction, M-S, varied from one region to the next, and the ranking order for the permeability was jejunum < ileum < colon for both the human and rat intestine. Passive diffusion was found to be the major membrane transport mechanism.

Intestinal efflux of LAs

Using the calcein AM assay (Eneroth et al 2001) it was found that ropivacaine and lidocaine caused retention of calcein in Caco-2VCR25 cells, implying that local anaesthetic compounds, such as ropivacaine, lidocaine and bupivacaine might be substrates for the P-glycoprotein. In addition, functional features of local anaesthetic compounds have previously been shown to be associated with Pgp activity (Seelig 1998).

Efflux ratios were studied in the Ussing chamber (pH 7.4) using excised tissue and the permeability ratios, P_{app} (S-M)/(M-S), were found to be 2.3, 1.8 and 3.0 for ropivacaine, lidocaine and bupivacaine, respectively, in rat jejunum (Paper I). This indicated that an efflux transporter, such as Pgp, might be involved in the intestinal permeability of LAs in the rat. In Paper IV, an efflux ratio of ropivacaine S-M/M-S at 50 μM of approximately two was demonstrated in jejunum, but it was not statistically significant. There was also an efflux ratio of approximately three for the ropivacaine metabolite PPX in rat jejunum, although that was not considered to be significant.

For human intestine, there were no evidence of involvement of any efflux transporters for either ropivacaine or PPX permeability in any of the intestinal regions as shown with permeability ratios S-M / M-S and addition of inhibitors (paper II). It has previously been shown that efflux ratios could differ between rat and human and that the rat could have a more pronounced drug efflux (Makhey et al 1998).

In situ single-pass perfusions of rat jejunum were performed to evaluate the relevance of the efflux ratios found in the Ussing chamber studies. The jejunal effective permeability (P_{eff}) using the *in situ* single-pass perfusion did not differ between 0.5 and 500 µM of LA, indicating that the permeability of the LAs was not concentration dependent. In addition, efflux inhibitors did not affect the permeability, either in the single-pass perfusion model, nor the Ussing chamber model.

Intestinal efflux mediated by an efflux transporter, such as Pgp, is probably not relevant to, or is only a minor influence on the intestinal absorption process of the three LAs and PPX. It is unlikely to affect the rate or extent of the intestinal absorption of any of these compounds possibly because the passive diffusion is more rapid than the efflux transport or the transporter could get saturated.

The impact of mucosal pH

In the Ussing chamber studies in Paper I, conducted at a mucosal pH of 6.5, the permeability exhibited significant S-M / M-S ratios of 17, 7.5 and 6.2 ($p<0.0001$) for ropivacaine, lidocaine and bupivacaine, respectively. With a mucosal pH of 7.4, the permeability ratio decreased to 2.3, 1.8 and 3.0 for ropivacaine, lidocaine and bupivacaine, respectively. This is in accordance with the pH-partitioning hypothesis for passive diffusion of weak bases with a pKa within the physiological range. According to the pKa-values for these drugs, the proportion of the unionized form of the LAs could be expected to increase from 5.9 to 33% for lidocaine and from 2.4 to 17% for ropivacaine and bupivacaine when the mucosal buffer pH was increased from 6.5 to 7.4. When the pH was increased, the increase in P_{app} was smaller than that of the fraction of unionized drug, suggesting that an acid microclimate may still have remained at the mucosal surface, causing a deviation from the pH partition hypothesis (Hogben et al 1959; Lucas 1983).

There are reports demonstrating that pH-changes within the physiological range does not affect the Pgp activity (Altenberg et al 1993; Borst & Elferink 2002; Neuhoff et al 2002). Therefore, the decreased efflux ratio at an apical pH of 7.4 was probably not attributed to decreased Pgp activity.

Correlation of intestinal permeabilities

Data from absorption models, such as the rat intestinal permeability (P_{app}) in Ussing chambers, rat *in situ* permeability (P_{eff}) and the Caco-2 cell permeability (P_{app}) have been shown to correlate well with the human *in vivo* intestinal permeability (P_{eff}) using the Loc-I-Gut® technique for drugs for which passive diffusion is the main absorption mechanism (Lennernäs et al., 1997; Fagerholm et al., 1996; Lennernäs et al., 1996). Incorporating the results from Paper I with published data from *in situ* single-pass perfusions and Ussing chamber studies, the permeability coefficients for the two absorption models were found to be highly correlated (r^2=0.9) (Figure 5)(Fagerholm et al 1996; Lennernäs 1997; Ungell et al 1998; Fagerholm et al 1999).

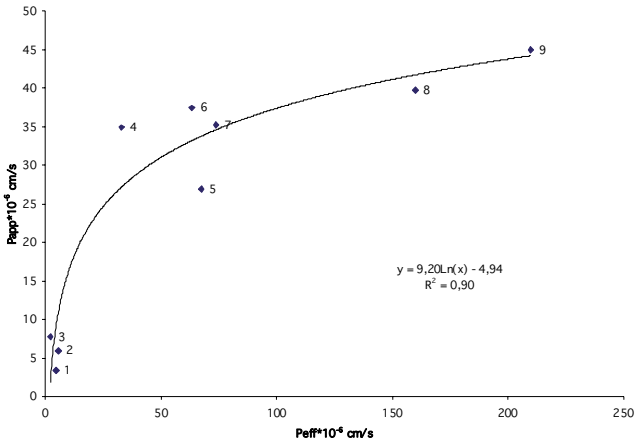

Figure 5. Correlation between in *vitro* permeability (P_{app}) and *in situ* permeability (P_{eff}) in the rat jejunum from Ussing chamber and by single-pass perfusion, respectively. The numbers represent the following compounds: 1. Terbutaline[a,b]; 2. Atenolol[a,b]; 3. Creatinine[c,b]; 4. Metoprolol[a,b]; 5. Bupivacaine; 6. Lidocaine; 7. Ropivacaine; 8. Antipyrine[a,b]; 9. Naproxen[a,d]

[a] P_{eff} value taken from Fagerholm et al 1996

[b] P_{app} value taken from Ungell et al 1998

[c] P_{eff} value taken from Fagerholm et al 1999

[d] P_{app} value taken from Lennernäs et al 1997

The rat has been recommended as a good model for the prediction of human intestinal passive permeability, including the regional perspective (Fagerholm et al 1996; Ungell et al 1998; Cao et al 2006). Ropivacaine and PPX absorptive permeability (P_{app} M-S), varied regionally in the order jejunum < ileum< colon in human intestine. The corresponding permeability for the rat exhibited a similar pattern. However, whilst the intestinal permeability of ropivacaine, P_{app} M- S, obtained from the Ussing chamber measure-

ments was comparable for rat and human jejunum (approx $1.5 \cdot 10^{-5}$ cm/s), for PPX, P_{app} was about 3.2 times higher in the rat. Using the intestinal perfusion technique, the jejunal permeability for passively absorbed drugs has been shown to be approximately 3.6 times higher in humans than rats (Fagerholm et al 1996). However, these results indicate that the difference between human and rat jejunal permeability is probably not solely a species difference, at least for ropivacaine and PPX, but could also be attributable to methodological differences.

Regional variations in intestinal metabolism

In human *in vivo* studies, ropivacaine underwent almost complete metabolism, with the main enzyme being CYP1A2, followed by CYP3A4 (Arlander et al 1998). PPX is the N-dealkylated metabolite of ropivacaine; it has been shown that PPX is metabolised by CYP3A4 in human hepatocytes (Ekström & Gunnarsson 1996). As CYP1A2 is expressed only weakly in human intestine (de Waziers et al 1990), CYP3A4 is the most important enzyme for metabolism of ropivacaine in the small intestine. Consequently, PPX was the only metabolite found in human intestinal Ussing samples. PPX formation was twice as great in human jejunum than in the ileum (Paper III), which is in agreement with previous studies on the metabolic activity of CYP3A4 (Paine et al 1997). In samples with colon, no PPX could be quantified as CYP3A4 is not expressed in human colon (de Waziers et al 1990; Zhang et al 1999). The metabolite formation using rat intestine was very similar to that in humans, with the highest formation rate being in the jejunum followed by the ileum. However, in the rat, PPX was also formed in the colon, as found with the Ussing chamber, as well as from microsome data (Paper IV). From Ussing chamber experiments on the rat the formation rate of 3-OH-ropivacaine could be calculated with acceptable accuracy, and was found to be approximately 60 times lower than the corresponding PPX formation rate. The formation of 4-OH was too low to be calculated. Metabolism differences between rat and human in a quantitative, qualitative and regional perspective is important to be aware of when rat is used as a model for human permeability and metabolism (Cao et al 2006).

The magnitude determined for the metabolism of ropivacaine in the different intestinal regions was consistent for the Ussing chamber measurements and the values found for microsomes. This indicates that the results for Ussing chambers and microsomes correlate well with regard to regional metabolism in the rat intestine.

Ropivacaine metabolism in rat intestinal microsomes

Ropivacaine was metabolized to PPX, 3-OH-ropivacaine and 4-OH-ropivacaine in rat intestinal microsomes (Paper IV). PPX was the main metabolite formed, and the PPX formation varied in the order jejunum >ileum > duodenum > colon, which was comparable to the results for metabolism obtained from the Ussing chamber. K_m values (assuming Michaelis-Menten kinetics) for the formation of PPX were approximately 3.5 mM, and for 3-OH-ropivacaine and 4-OH-ropivacaine, 1 mM. Cimetidine, troleandomycin, furafylline and quinidine were used as inhibitors for cyp2c, cyp3a, cyp1a and cyp2d, respectively, to identify enzymes involved in ropivacaine intestinal metabolism in the rat.

In rat liver microsomes, cyp3a2 and cyp2c11 mediate the PPX formation from ropivacaine (Oda et al 1995), but in the intestine, PPX was formed, to some extent, by cyp2c and cyp2d and only to a minor extent by cyp3a (Paper IV). Cyp2d involvement in the dealkylation of ropivacaine would be in agreement with the similar compound lidocaine, for which the deethylated metabolite (Meg-X) is mediated by cyp2d in rat liver. Cyp2d has also been shown to be involved in the hydroxylation of lidocaine (Wan et al 1997) of which there were indications in our study regarding 3-OH-ropivacaine and 4-OH-ropivacaine formation. However, the PPX formation was not very strongly inhibited, and therefore it cannot be excluded that another enzymes is involved in PPX formation. It is also possible that troleandomycin is a weak inhibitor for the cyp3a isoforms present in the rat intestine (Tanaka et al 2006).

Ropivacaine metabolite extrusion

Apart from the mentioned theory on the cooperativity of CYP3A4 and Pgp by enhancing the metabolism by reduction of the presentation rate, it has been proposed that Pgp is mediating the transport of CYP3A4 metabolites to be extruded from the mucosal side of the enterocyte (Hochman et al 2001). CYP3A4-mediated metabolism of ropivacaine and transport of the N-deethylated metabolite PPX was investigated using excised tissue from human jejunum and ileum (Paper II) and from rat jejunum, ileum and colon (Paper IV). PPX could not be detected in human colon. Intracellularly formed PPX was predominantly extruded from the intestinal segment to the mucosal chamber when ropivacaine was transported in the M-S direction for both rat and human intestine (Figures 6 and 7). When investigating the extrusion of metabolite to the different compartments of the Ussing chamber, the extrusion rates (calculated with Eq. 3) to the mucosal compartment (muc) and the serosal compartment (ser) were determined. Human jejunum had an M-S (muc/ser) ratio of 8.1, which means that when ropivacaine is

passing through the intestinal tissue in the direction M-S, the extrusion of the intracellularly formed metabolite PPX is 8.1 times higher to the mucosal compartment than to the serosal compartment. This is indicative of polarized metabolite extrusion. For the human ileum the M-S (muc/ser) was 3.8 and, for the rat intestine, the corresponding M-S (muc/ser) ratios were 25, 15 and 5 for the jejunum, ileum and colon, respectively.

An examination was also made of how important the transport direction of the parent compound was for this metabolite polarization. The extrusion towards the mucosal compartment was 13 times higher when ropivacaine was transported in the direction M-S rather than S-M for human ileum. The corresponding value for jejunum was 2.7, suggesting that the metabolite extrusion in ileum was more dependent on the ropivacaine transport route than it was in jejunum. For rat intestine, this route dependence was not as significant. Other compounds that have shown polarised extrusion of metabolites are midazolam, cyclosporine, sirolimus, indinavir and verapamil (Gan et al 1996; Schmiedlin-Ren et al 1997; Lampen et al 1998; Hochman et al 2001; Johnson et al 2001b).

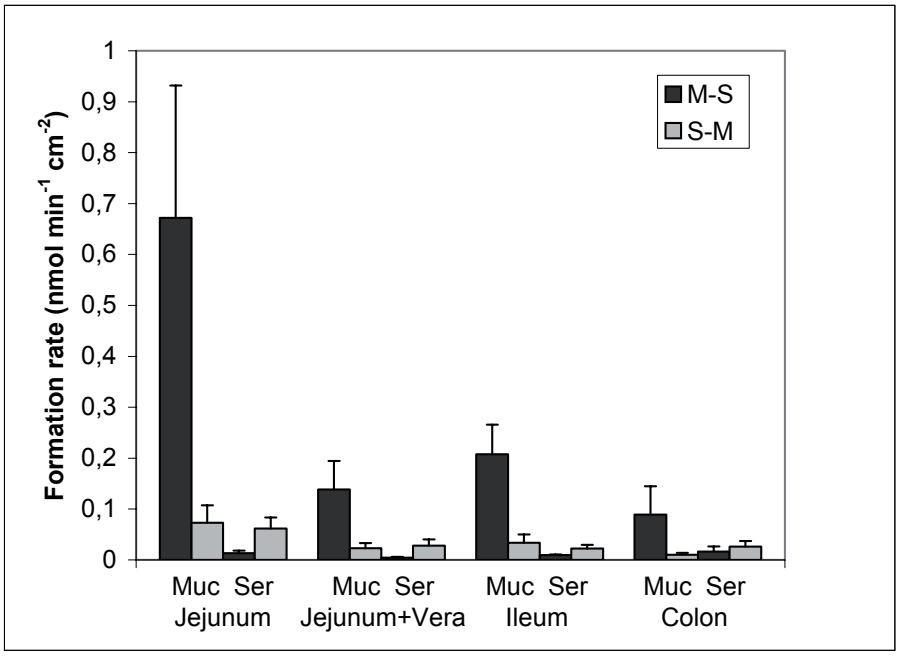

Figure 6. The formation rate of PPX from 500µM ropivacaine in rat intestine to mucosal (muc) and serosal (ser) Ussing chamber compartments.

Ketoconazole (10 µM) and verapamil (500 µM) inhibit Pgp and CYP3A enzymes, and they inhibited the PPX formation in intestinal tissues. Accordingly, the polarized transport of the metabolite was probably not only caused by Pgp-mediated activity because the polarized extrusion pattern was present with also with added verapamil. The polarization could reflect the distance from the CYP3A4 enzyme to the apical membrane, as it has been suggested that the CYP3A4 enzyme is polarized towards the apical side in the intracellular space (Watkins 1997; Cummins et al 2001).

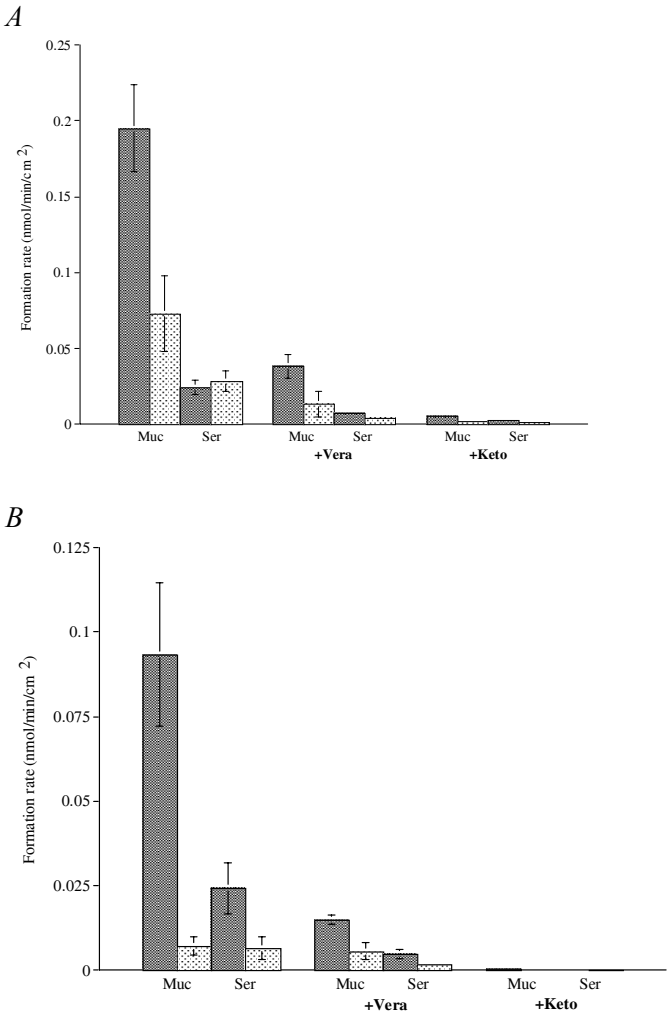

Figure 7. The PPX metabolite extrusion rate in human (A) jejunum and (B) ileum as ropivacaine (500 µM) is transported in the direction of mucosa to serosa (▩) and serosa to mucosa (▨). Inhibition of PPX extrusion rate to mucosal (muc) or serosal (ser) compartments with added verapamil (Vera) (500 µM) and ketoconazole (Keto) (10 µM) in both compartments, mean ± SEM.

Pgp, MRP1, MRP2 and CYP3A4 expression in human jejunum, ileum and colon

Tissues from human jejunum, ileum and colon were analyzed to determine the mRNA and protein distribution along the human intestine (Paper III). The mRNA levels were determined using RT-PCR and the protein levels with Western blot analysis. The jejunum had the highest mRNA expression level of CYP3A4 and MRP2, and followed the pattern, jejunum >ileum > colon. This intestinal pattern for the regional distribution of MRP2 has previously been reported in both rat and dog (Gotoh et al 2000; Conrad et al 2001). For CYP3A4, the distribution correlated well with CYP3A4 metabolising activity in human intestine, previously shown for midazolam (Paine et al 1997) and for ropivacaine (Paper II).

For Pgp, a similar pattern of mRNA regional distribution was seen as for CYP3A4 and MRP2, but the regional differences were not statistically significant because of a high degree of inter-individual variability. Some contradictory results have been obtained for the regional distribution of Pgp. In earlier studies, it was reported that Pgp expression levels increase towards the colon (Fojo et al 1987; Fricker et al 1996; Mouly & Paine 2003), whereas more recent studies indicates the opposite distribution (Nakamura et al 2002; Englund 2005; Zimmermann et al 2005). It would be reasonable for Pgp and CYP3A4 to have the same regional distribution in the intestine as their substrate specificity overlaps almost completely. It has also been suggested that they are co-regulated (Synold et al 2001) and cooperate in apical recycling (Benet & Cummins 2001). Finally, there was a noteworthy indication in Paper III, that there could be common regulatory mechanism between CYP3A4, Pgp and MRP2, as the same individual had the highest expression of CYP3A4, Pgp and MRP2. The reason for this is not known, as to our knowledge, it could not be caused by any medical treatment of the individual.

MRP1, a basolaterally located transporter unlike Pgp and MRP2, did not exhibit any regional variation in mRNA expression. The function of this transporter is not known, but it is probably not involved in the efflux of drugs.

mRNA and protein level correlation for Pgp, MRP1, MRP2 and CYP3A4

For CYP3A4, the mRNA and protein levels were significantly correlated ($r = 0.779$, $n = 13$ and $p = 0.0017$), for MRP2 the correlation was not as good ($r = 0.690$, $n = 12$, $p = 0.0130$), and, for Pgp, there was no correlation at all ($r = 0.115$, $n = 13$ and $p = 0.708$)(Paper III)(Figure 8). For Pgp the inter-individual variability at the protein level was even more extensive than for

mRNA and, consequently, no region-dependent expression patterns could be established. It has previously been observed that correlations of mRNA, protein and enzyme activity are sometimes lacking for enzymes, such as CYP3A and CYP2C in the liver and intestine, which may be related to mRNA translation and/or tissue processing (Lown et al 1994; Läpple et al 2003). As shown in Paper III, this could also be valid for the expression and activity of ABC transporters, as the absence of correlation was even more obvious for the transporters than for the CYP3A4 enzyme. CYP3A4 expression could be detected in colon although there were no indications of CYP3A4 activity in the colon in Paper II. The MRP1 protein level was not determined because the expression was too low for Western blot analysis to give a reliable measure, and therefore no correlation of MRP1 could be established.

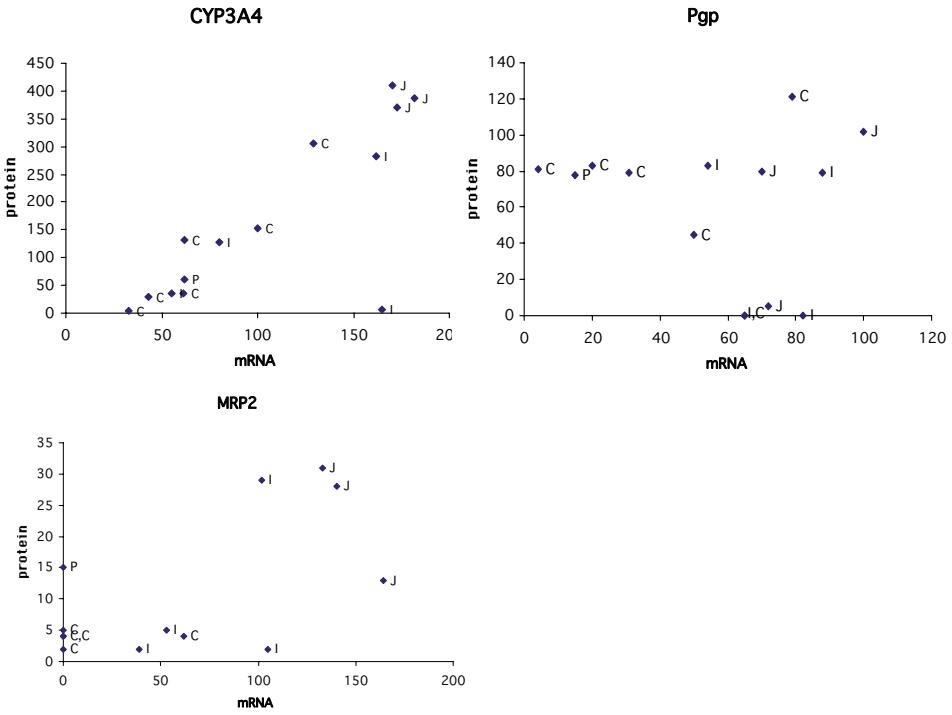

Figure 8. Correlation between mRNA and protein of CYP3A4, Pgp and MRP2 for the jejunum (J), ileum (I), proximal colon (P) and distal colon (C). mRNA levels are given as a percentage of the optical density (OD) of villin and the protein levels are given relative to a standard sample.

Conclusions

The intestinal permeability of the model compounds ropivacaine, lidocaine and bupivacaine was classified as high. Passive diffusion was probably the main membrane transport mechanism, whereas carrier-mediated intestinal efflux may occur to a small degree without affecting the intestinal absorption.

The permeability values obtained from *in situ* single-pass perfusion and the Ussing chamber correlated well with previously obtained permeability data in rat jejunum. Permeability efflux ratios (S-M / M-S) of the model compounds obtained with the Ussing chamber did not correspond to the results obtained with the perfusion technique.

The regional permeability (P_{app}) of ropivacaine and PPX increased in the order jejunum < ileum < colon in both the human and the rat, as would be expected for high permeability compounds.

Both CYP3A4 metabolism and metabolite extrusion to the mucosal compartment were more extensive in the human jejunum than the human ileum. The polarized extrusion of the metabolite PPX from rat and human excised intestine was not explained by any active transport mediated by Pgp.

The expression levels of CYP3A4, Pgp and MRP2 demonstrated a regional difference varying in the order jejunum > ileum > colon. The mRNA and protein levels were significantly correlated for CYP3A4 but correlated to a lesser extent for MRP2. However, they were not correlated at all for Pgp.

The regional metabolism of ropivacaine in rat intestine was consistent for the measurements using microsomes and the Ussing chamber and varied in the order jejunum > ileum > duodenum > colon.

The metabolism of ropivacaine to PPX in human intestine was mediated by CYP3A4, whereas in the rat jejunum it was not mediated by cyp3a, but rather by cyp2d and cyp2c.

PPX was formed in the rat colon but not in the human colon.

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