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The role of intestinal transporters in limiting bioavailability of diuretics

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The Role of Intestinal Transporters in Limiting Bioavailability of Diuretics: Studies with Two Model Compounds, Amiloride and Furosemide

by

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DISSERTATION

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To Debra, Kayla and Lilia

with Love

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Shawn Daniel Flanagan June, 1999

Abstract

The Role of Intestinal Transporters in Limiting Bioavailability of Diuretics:

Studies with Two Model Compounds, Amiloride and Furosemide

Shawn Daniel Flanagan

Transporters such as P-glycoprotein (P-gp) can play a role in limiting drug bioavailability. Diuretics are substrates for renal secretory transporters, frequently also exhibiting low bioavailability. The possibility that these compounds may be secreted in the small intestine was studied using two model compounds, amiloride and furosemide, in two *in vitro* absorption models. Both amiloride and furosemide showed net secretion from excised rat jejunum and Caco-2 cells. Amiloride secretion from Caco-2 cells did not demonstrate temperature or concentration dependence, or sensitivity to chemical inhibition. Furosemide secretion was saturable, temperature and energy dependent and subject to, (presumably competitive) inhibition by bumetanide, cyclosporine, indomethacin, probenecid, sulfinpyrazone and vinblastine. A specific transport protein responsible for furosemide secretion could not be identified using polarized epithelial cell cultures transfected with three well characterized human transport proteins. Permeation of furosemide across P-gp and the Multidrug Resistance-associated Proteins (MRP1 and MRP2) expressing cell lines were the same as for the non-transfected wild-type cells. Preliminary results from a clinical study (three of nine subjects) showed that oral

indomethacin (100 mg) caused a decrease in furosemide bioavailability. The area under the curve (AUC) for intravenous furosemide administration was increased to a greater extent than the orally administered furosemide, with the same oral indomethacin regimen.

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Chapter 1

Introduction

1.1 Transporters

Intestinal drug absorption was until recently thought to occur primarily by simple passive diffusion through cell membranes (transcellular). Earlier findings that the small intestine could secrete drugs in an active (George *et al.*, 1979) or saturable (Turnheim and Lauterbach, 1980) manner were largely viewed as exceptions, and carrier-mediated transport was primarily studied for nutrients such as amino acids (Christenson et al., 1963). Paracellular transport (discussed further in the following section) has long been suspected for very small hydrophilic molecules whose absorption was greater than would be predicted based on physicochemical properties (Crone and Keen, 1969). Studies with larger hydrophilic compounds led to some of the first observations that specific transport proteins could affect drug absorption. Two examples include salicylic acid by the monocarboxylic acid transporter (Porter *et al.*, 1985) and cephradine by the dipeptide transport system (Okana *et al.*, 1986). It was not until an efflux transporter associated with resistance to chemotherapeutics (P-glycoprotein) was localized ton the apical surface of human jejunum by Thiebaut *et al.* (1987) that lower than expected absorption could be explained (Hunter *et al.*, 1993a) in a broader context. This chapter will focus primarily on transporters that may limit absorption.

Permeability-glycoprotein (P-glycoprotein, or P-gp) was originally thought to increase membrane permeability so that toxic therapeutic agents could leak back out of cancer

cells (Juliano and Ling, 1976), but this protein was later found to be an efflux pump. Because of its overexpression in many types of cancer P-gp has been extensively studied and characterized, and is also referred to as the multidrug resistance (MDR) protein. A member of the ATP-binding cassette (ABC) transporter family, characterized by having two ATP-binding sites and twelve transmembrane domains, its low resolution (2.5 nm) structure has been determined by electron microscopy and image analysis (Rosenberg *et al.*, 1997).

P-gp is found in normal tissues as well, serving essentially a protective role for the body in such organs as the liver, kidney, pancreas, small intestine, placenta, brain and testis. Its ubiquitous presence in the adrenal glands is less clear, but may indicate a role in steroid secretion (Gottesman and Pastan, 1993). The substrate specificity of P-gp is extremely broad, possibly reflecting more than one binding site as substrates include diverse chemical structures, such as calcium channel blockers, calmodulin antagonists, anthracycline and vinca alkyloid analogues, as well as steroids and hormonal analogues (Ford and Hait, 1990). A detailed summary is provided by Wacher *et al.* (1995) who point out the surprising overlap of drugs interacting with both the P-gp transporter and the body's principle drug metabolizing enzyme, Cytochrome P450 3A4 (CYP3A4).

More recently another transporter has been identified in the ABC family of proteins that also confers resistance to the tumor cells that express it. Discovered and named MDR-associated protein (MRP) by Cole *et al.* (1992), this transporter has been characterized as an efflux pump for drugs (Zaman *et al.*, 1994) and their glutathione conjugates (Jedlitschy *et al.*, 1994). MRP is functionally similar to P-gp, transporting many of the same substrates such as vinblastine, cyclosporine, and verapamil (Holló *et al.*, 1996), but only distantly related to P-gp with about 15% homology (Lautier *et al.*, 1996). Unlike P-gp which preferentially transports lipophilic or weakly basic substrates, MRP prefers

anionic compounds and products of phase II metabolism such as glutathione conjugates (Heijn *et al.*, 1997; Shen *et al.*, 1996). Another difference is that in humans there is purported to be only one P-gp that transports drugs, whereas MRP is actually a family of proteins with at least six members (Kool *et al.*, 1997).

MRP1 is the only member of this family that has been shown to be associated with multi-drug resistance (Kool *et al.*, 1997) and is found in a variety of solid tumors as well as normal tissue including small intestine and liver (Kruh *et al.*, 1995). Its expression in the liver was found to be very low (Paulusma *et al.*, 1996), unlike that of a homologous transporter (MRP2, also termed the canalicular multispecific organic anion transporter, cMOAT) that is found almost exclusively in the liver and in lesser amounts in the small intestine and other tissues. Its transport properties had been well characterized by Ishikawa (1992) and Oude Elferink and Jansen (1994). Early evidence suggests that MRP3 is like MRP2, MRP4 is rare, MRP5 is like MRP1, and the role of MRP6 is still a mystery at this time. Homology between family members suggests that all function as GS-X transporters (Kool *et al.*, 1997).

The lung resistance protein (LRP) is another protein associated with multidrug resistance found in normal (including digestive tract) and cancerous tissues (Izquierdo *et al.*, 1996). This vault protein of unknown function has been speculated to be involved in drug transport since it serves as a better prognosticator for multidrug resistant cancer strains than either P-gp or MRP, but its expression does not directly result in multidrug resistance (Sugawara *et al.*, 1997). Thus far, LRP has only been found in the cytosol with no evidence of either transport or sequestering of drugs (Borst *et al.*, 1997).

Two other systems capable of transporting a variety of drugs are the organic anion and cation transporters (OAT and OCT, respectively). These transporters have long been

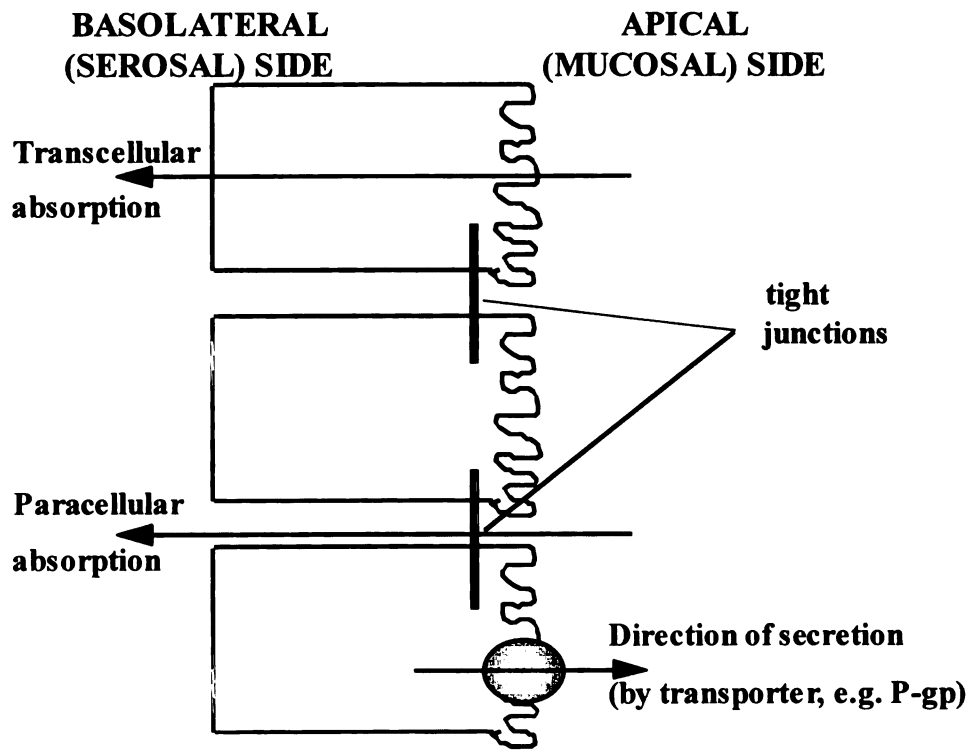
studied in the kidney where they are known to function in both the secretion and reabsorption of anionic and cationic endo- and xenobiotics. Renal drug secretion of anions and cations involves both basolateral uptake and brush border efflux transporters and anionic reabsorption across the brush border can occur as well under certain conditions. Substrate specificity varies among the various isoforms, but is in general quite broad (Bendayan, 1996). There is also some overlap, with cations such as cimetidine interacting with organic anions (Bendayan, 1996), and the anions probenecid and furosemide interacting with cations such as N-methyl nicotinamide (Ott *et al.*, 1990). In fact, the number of "bisubstrates" interacting with both organic anion and cation transport systems is extensive as noted in a two-part study by Ullrich *et al.* (1993 a and b).

These transporters have been less well studied in other tissues. A rabbit organic cation transporter (rbOCT1) has been found in liver and small intestine by both RT-PCR and Northern blot analysis (Terashita *et al.*, 1998). Similarly, a rat transporter (OCT3) isolated from placental tissue was found to be moderately expressed in the intestine (Kekuda *et al.*, 1998). Various functional studies (reviewed by Koepsell, 1998) have implied the presence of other organic cation transporters in the small intestine. For example, the cation guanidine was found to be transported in rabbit brush border membrane vesicles in a manner that was not affected by typical OCT inhibitors (Miyamoto *et al.*, 1988). The secretion of anions has been observed to occur in the small intestine (Saitoh et al., 1996) but specific transport proteins have not yet been identified. At least one organic anion transporter (OAT-K1) is known to not be present in the small intestine (Masuda *et al.*, 1997).

The field of intestinal drug transport is still in its infancy. We can expect in the near future to not only determine which membranes contain known transporters, but can also

look forward to the discovery of many new transporters. At present we can best characterize transport functionally by studying the directional permeation of drugs. Substrates for a secretory transporter, such as P-gp, will show polarized transport *in vitro,* with basal to apical (secretory) fluxes greater than those in the apical to basal (absorptive) direction. A diagram showing this nomenclature for a row of enterocytes is given in fig. 1-1.

Figure 1-1: Diagram of intestinal routes of absorption and nomenclature.



1.2 Route of Absorption (Transcellular *vs.* Paracellular)

Most orally administered drugs reach the general circulation through the lining of the small intestine as a result of its large absorptive surface area. Drugs in solution must pass

either through the lipid bilayer membrane surrounding the cell (trancellular), or through the tight junctions or *zonula occludens* between the cells (paracellular), as described in fig. 1-1. Certain drug features such as size (MW) and relative lipophilicity (log P) can help determine the route of absorption. Hydrophilic compounds such as glucose and amino acids as well as some drugs that cannot penetrate the cell membrane are taken up into the cell by carrier proteins (e.g. dipeptide, bile acid, and phosphate transporters) in a facilitated or energy dependent manner, or by endocytosis. Small lipophilic compounds that can freely diffuse across lipids tend to go by the transcellular passive route. The paracellular pathway tends to prefer small hydrophilic molecules and can be altered by nutrients, e.g. glucose, and amino acids (Sadowski and Meddings, 1993), calcium chelation (Boulenc *et al.*, 1993; Noach *et al.*, 1993) and membrane permabilizers such as palmitoyl carnitine (Hochman *et al.*, 1994). In addition, some drugs are capable of increasing (verapamil, Sakai *et al.*, 1994) or decreasing (ranitidine, Gan *et al.*, 1998) paracellular permeation. The ability of a transporter to effectively limit bioavailability depends to some extent on the percent of the total drug permeation going through the transcellular route. The pathway that a drug molecule takes in entering the body can therefore play a role in determining the importance of any transport observed *in vitro*.

1.3 Diuretics

Diuretics are an important class of compounds used for a variety of indications including both edemetous states, e.g. congestive heart failure, and non-edemetous states, e.g. essential hypertension. Most diuretics are actively secreted in the kidney (predominately as unchanged drug) and remove water indirectly, primarily by increasing renal secretion of ions such as sodium. It is the removal of sodium "natriuresis" that results in the observed diuretic effect of increased urine volume (Ives and Warnock, 1995). All classes of diuretics, except the osmotic that are essentially inert non-absorbable compounds such as mannitol, are actively secreted in the kidney predominately as unchanged drug.

Bioavailability in general is quite low as summarized in table 1-1 (modified from Jackson, 1996). The fact that diuretics are secreted by renal transporters has lead to the hypothesis that they may also be actively secreted back into the small intestine, by transporters sharing homologous function and/or structure.

Table 1-1: Variable and generally poor bioavailability of diuretics.

Class / *Mechanism of diuresis*	Drugs	Oral Bioavailability
Weak/Potassium sparing	Amiloride	15-25%
Blocks sodium channel	Triamterene	30-70%
Moderate/Thiazide	Chlorothiazide	15-25%
Blocks sodium/chloride symport	Hydrochlorothiazide	65-75%
(also inhibits carbonic anhydrase)	Chlorthalidone	60-70%
High-ceiling/Loop	Furosemide	11-90%
Inhibits coupled $Na^+/K^+/2Cl^-$ transporter	Bumetanide	59-89%

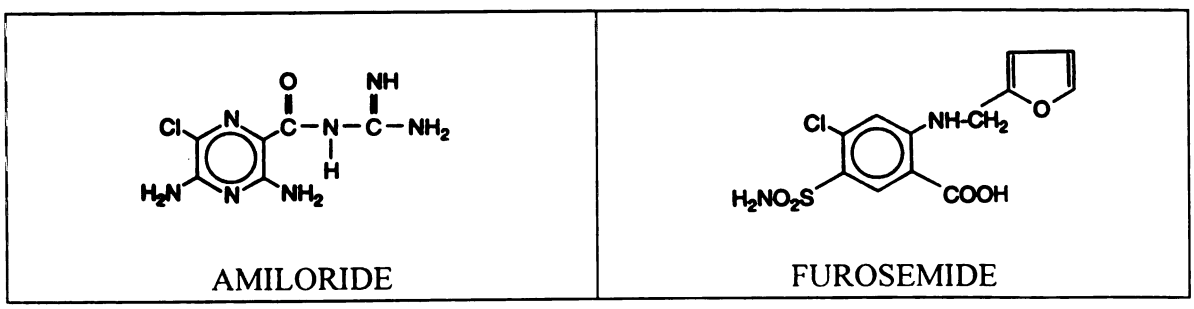
There is some evidence suggesting that diuretics may be secreted in the intestine. Direct evidence is provided by the over eight-fold net secretion of chlorothiazide by Caco-2 cells as observed by Pade and Stavchansky (1998). Secretion of celiprolol by Caco-2 cells was inhibited by the known P-glycoprotein substrates vinblastine, verapamil and nifedipine, as well as by beta-blockers and the diuretics acetazolamide, chlorothalidone, and hydrochlorothiazide (Karlsson, et al., 1993), suggesting that these diuretics may also

be P-gp substrates. Literature specific to amiloride and furosemide provided some of the basis for their selection as model compounds and is summarized in the following section.

1.4 Selection of Amiloride and Furosemide as Model Diuretics

A number of factors led to the selection of furosemide and amiloride as model compounds. They both have poor and variable oral bioavailability. According to the chapter on diuretics in the most recent edition of Goodman and Gilman (Jackson, 1996), furosemide has 11-90% oral bioavailability and amiloride has between 15-25%, as noted in table 1-1. They are structurally (see fig. 1-2) and pharmacologically diverse, acting on different targets in different regions of the nephron thereby exerting different magnitudes of diuresis. They both possess good fluorphores providing ease of chromatographic detection, and they differ markedly in pKa. This last point is significant as they carry opposite charges at physiological pH, and are likely to be substrates for different transporters. Amiloride (pKa 8.5) is likely to be a preferred substrate of OCT or P-gp, whereas furosemide with its pKa of 3.9 is a more likely candidate for OAT or MRP transport. It will be necessary to characterize paracellular permeation for these charged molecules if efflux intestinal transporters are to play an important role in limiting their absorption.

Figure 1-2: Chemical structures of amiloride and furosemide.



Active intestinal transport of these diuretics could explain their poor oral bioavailability. There is some evidence in the literature supporting this hypothesis. Amiloride has been known to provide clinical benefit to cystic fibrosis patients, possibly through blockage of MDR-type efflux pump in a *Pseudomonas* species associated with the disease (Cohn *et al.*, 1995). The suceptibility of this organism to antibiotic treatment increases in the presence of amiloride, possibly by limiting the antibiotic efflux. Amiloride itself may be a substrate for this transporter in addition to its role as an inhibitor.

Several researchers have speculated that furosemide is secreted in the small intestine (Branch, 1983; Lee and Chiou, 1983; Valentine *et al.*, 1986; Verbeeck *et al.*, 1982). Intestinal secretion of furosemide was not an important route of elimination for intravenous furosemide (Arimori and Nakano, 1988; Valentine *et al.*, 1986). However, because of the location of the transporters on the apical side of the enterocytes, secretion could play a role in limiting bioavailability of an oral dose. Pade and Stavchansky (1998) found net secretion of furosemide by Caco-2 cells which we confirmed in Caco-2 and rat intestine (Flanagan and Benet, 1999, included as chapter 4 of this thesis). This secretion could explain the poor oral absorption of furosemide.

1.5 Specific Objectives

The overall objectives of this thesis research are summarized below.

1. To determine if the permeation of amiloride and furosemide through excised or cultured intestinal tissue is greater in the direction of secretion than absorption.
2. To test if observed net secretion of amiloride and furosemide is the result of active secretory transport.
3. To characterize which transporters may be responsible for net secretion of amiloride and furosemide.

4. To determine the major transport pathway for amiloride and furosemide, i.e. transcellular *vs*. paracellular.
5. Determine if coadministration of a transport inhibitor with amiloride or furosemide will result in increased bioavailability and/or decreased variability of the diuretic.

Chapter 2

In Vitro Models For Assessing Intestinal Absorption

2.1 Overview of *in vitro* models

2.1.1 Excised tissue

Excised intestinal tissue from a variety of animal species has been used since the 1950's to study intestinal absorption. Two of the most widely used and long-lasting techniques using excised tissue are Ussing chambers (Ussing and Zerahn, 1951; Lennernäs, 1998) and the everted sac (Wilson and Wiseman, 1954; Barthe *et al.*, 1998). Both of these systems allow measurement of diffusion across intestinal tissue. Other studies have looked at measurement of uptake in slices or rings (Shaw *et al.*, 1983). All three methods are good in that the tissue, itself from the main absorptive area of the digestive tract, is left intact. Studies requiring chambers are perhaps the most costly in terms of initial expenses and sacs in terms of number of animals needed. The easiest to use and cheapest type being the rings which are not without their own limitations as uptake is not easily determined for non-radiolabeled compounds (Stewart *et al.*, 1995).

2.1.2 Cellular and subcellular fractions

Membrane vesicles and isolated cells have been used to study intestinal transport. Osiecka *et al.* (1985) found membrane vesicles and isolated cells to be unacceptable models for predicting human intestinal permeability. Another article from the same group (Porter *et al.*, 1985) showed that isolated cells were severely handicapped by their

lack of polarity and did not correlate as well to human absorption as the excised ring model. It should be noted that brush border membrane vesicles are an important tool and have been used by many groups successfully to study aspects of intestinal transport (Hsing *et al.*, 1992; Miyamota *et al.*, 1988; Polache *et al.*, 1993; Sinko *et al.*, 1995; Tamai *et al.*, 1997).

2.1.3 Cultured cells

A number of cultured epithelial cells have been used for absorption screening. These cell lines and their tissue of origin are given in table 2-1, together with a tabulation of their use as absorption models based on the number of literature references. Canine or porcine kidney (Madin-Darby Canine Kidney [MDCK] and LLC-PK1, respectively) cells have mainly been useful only when transfected with transporters relevant to absorption such as P-gp. In fact all five of the drug related references cited for these cell lines in table 2-1 describe transfected cells. A recent report by Irvine and others (1999) suggested that MDCK cells are as good predictors of human intestinal absorption as Caco-2 cells. This fact taken together with the markedly reduced culture times required, which is approximately one-third of the required time for Caco-2 cells, may lead to their increased use. MDCK cells are also being used as a model for another tissue, the blood-brain-barrier, in spite of its renal origin.

The most widely used and accepted cell culture model for intestinal absorption is the adenocarcinoma (Caco-2) cell line. This colon carcinoma cell line has been shown to spontaneously differentiate *in vitro* to resemble polarized enterocytes of the small intestine complete with brush-border microvilli and associated digestive enzymes such as sucrase and aminopeptidase (Pinto *et al.*, 1983). Other human cell lines of colorectal origin, such as HT-29, TC-7 and T84, have seen limited use for absorption studies (table 2-1).

Table 2-1: Commonly used cell lines.

Origin	Cell line	Hits[A]	and "absorption" or "bioavailability[B]	Drug related[C]
Human colon	Caco-2	941	189 (20.1)	93
	HT29	606	18 (3.0)	2
	TC7	30	1 (3.3)	0
	T84	232	4 (1.7)	0
Canine kidney	MDCK	455	4 (0.9)	2
Porcine kidney	LLC-PK1	402	7 (1.7)	3
Rat intestine[D]	IEC-18	39	2 (5.1)	1
"rat" (general)	—	*86228*	*1551* (1.8)	*ND*

A. Number of Medline (1995-1999) hits using cell line name as keyword (searched 3/26/99).
B. Number of hits (and percentage) using cell line name and keyword "absorption" or "bioavailability".
C. Number of hits from B, related to drugs as determined by title and abstract.
D. Derived from rat ileal crypt cells.
ND Not Determined

The T84 cell line also spontaneously differentiates, but only to resemble normal colonic cells with an extremely high resistance and are not useful for drug transport. This cell line which lacks a differentiated brush border has been used to study passive transcellular drug flux (Brayden, 1997). Collett and coworkers (1996) found a subclone of HT-29 cells to be useful for predicting paracellular drug transport because of its slow development of resistance, approximating human intestine after 10 days. Caco-2 cells

were used by the same group a few years later, however, to show P-gp effects on two hydrophilic H_2-receptor antagonists (Collett *et al.*, 1999). This HT29-18C_1 subclone may see greater use since although it must be induced to differentiate, it does so quicker than Caco-2 cells, and also expresses goblet secreting mucous cells that are lacking in the Caco-2 model (Brayden, 1997). The TC-7 clone was found to have several advantages over the parental Caco-2 cells, namely increases and/or decreases in expression of various transporters and/or enzymes that made the clone more like human jejunum (Grès *et al.*, 1998). This cell line may see increased use, but still shares the biggest limitation of its parent, that is long culture times.

Recently, a 3-day Caco-2 system (Biocoat® Intestinal Epithelium Differentiation Environment, BIEDE) has been introduced and marketed by Becton Dickinson (Bedford, MA) to decrease the time and labor requirements of this model. These reduced culture times are the result of seeding the cells at very high densities onto fibrillar collagen coated membranes and then, using a variety of hormones and other chemicals, accelerate their attachment and differentiation. Permeability coefficients obtained with these cells were similar to values obtained with standard 21 day cultures for a wide range of passively absorbed compounds (Chong *et al.*, 1997). A functional comparison of transporters in the 21-day Caco-2 and 3-day BIEDE systems showed that only the bile-acid transporter was lacking in the 3-day system, but that other efflux and absorptive transport properties were similar (Sweetland and Polzer, 1998). Overall permeability and electrical resistance in the BIEDE systems is lower and more like human small intestine than for the cells grown for 21 days. Despite these results, this model has yet to become widely used. Cost may be a factor, as the BIEDE systems require both expensive media and membranes. In addition, the time savings in not having to maintain the 3-day systems for 3 weeks is partially offset by having to generate enough cells for the

extremely high seeding density, and performing more media changes than would normally be required during that initial short period.

A non-transformed small intestine-derived cell line, IEC-18, has recently been studied as a model for drug absorption, especially for studying the paracellular path of absorption (Duizer *et al.*, 1997 and 1998). These cells may only see limited use due to a number of limitations. They are isolated from ileal crypt cells rather than villous cells and have low differentiation (Duizer *et al.*, 1997). Other small intestinal cell lines are being developed currently. The benefit of a cell line originating from the tissue of interest is obvious, but as two recent abstracts from Per Artursson's group indicate, they have not yet surpassed other models such as Caco-2 cells (Engman *et al.*, 1998; Tavelin *et al.*, 1998).

Caco-2 cells are being altered by transfecting with the cDNA coding for various transporters or metabolic enzymes to make them more like the human intestine it mimics. Sometimes adding a nutient such as 1α,25-dihydroxyvitamin D_3 can increase expression levels of both CYP3A4 and P-gp (Schmiedlin-Ren *et al.*, 1997). Since P-gp is already higher in Caco-2 than human intestine, a better approach may be to directly transfect cDNA for 3A4, as done by Crespi *et al.* (1996). In addition to the low levels of 3A4, peptide transporters are also lacking or only weakly expressed in Caco-2 cells. The human intestinal oligopeptide transporter (hPepT1) cDNA has been transfected into Caco-2 as well as IEC-18 cells (Hsu et al., 1998).

A problem with transfecting Caco-2 cells is that these cells are already polyclonal and a different sub-population could be developed by selection. Comparisons between transfected and wild-type cells may not be valid for these cells, so if the goal is to elucidate an interaction with a particular transporter then other more homogenous cell lines are used. Two polarized kidney cell lines (LLC-PK1 and MDCK) have been used

for over expression of specific transporters, and these tranfected cell lines are freely available for use by academicians and may be licensed by industrial scientists. There are actually two different strains of MDCK cells, I and II, with the former being low passage number and high resistance and *vice versa* for the latter. Strain I cells have been transfected with P-gp (Pastan *et al.*, 1988) and both strains have been transfected with cMOAT (Evers *et al.*, 1998). In addition, P-gp (Schinkel *et al.*, 1995; Tanaka *et al.*, 1996) and MRP1 (Evers *et al.*, 1996) have been transfected into LLC-PK1 cells.

2.2 Selection of two absorption models

Since no *in vitro* model is without limitation, two different models were selected for these studies to provide better *in vivo* predictions. The models selected are perhaps the most widely used and accepted absorption models, excised rat intestine in modified Ussing diffusion chambers and Caco-2 cells grown on permeable filter supports. The rat diffusion chamber model (Grass and Sweetana, 1988) is a vast improvement over the simple and easy to use everted sac model. The main advantages of the Grass-Sweetana diffusion chamber include more uniform stirring (by oxygenating gas air-lift) and more accurate quantitatation. In addition, the diffusion chambers have a well defined opening of known area, whereas the surface area (an important term in flux determinations) is difficult to estimate and may vary considerably between everted gut studies. The donor and receiver volumes are equal and can be added to more precisely than the difficult to fill sacs with their vastly different internal and external volumes. The rat diffusion chamber model has been used to characterize passive transport via the paracellular and transcellular pathways, as well as active carrier mediated absorption (Swaan and Tukker, 1995) and P-gp mediated efflux (Saitoh and Aungst, 1995).

The Caco-2 model provides the most defined relationship of any *in vitro* model with human absorption (Stewart *et al.*, 1995). A major advantage of Caco-2 cells over excised tissue is that about ten-fold as many studies can be conducted with the same effort. An overview of the strengths and weaknesses of this model are given in table 2-2. Several researchers have characterized transport properties for this cell line and demonstrated it to be a highly useful model for absorption studies (Hidalgo *et al.*, 1989; Hilgers *et al.*, 1990) having few limitations (Delie and Rubas, 1997). The major limitations are too high a resistance to hydrophilic compounds (Collette *et al.*, 1996), overexpression of efflux transporters, and underexpression of both carrier mediated absorptive transporters (Lennernas, 1998) and key metabolizing enzymes such as P450 3A4 (Crespi *et al.*, 1996). If Caco-2 cell permeation was an assessment criteria for drug absorption, then some drugs would have never been developed. The well absorbed (greater than 90%) and highly useful drugs, amoxicillin and cephalexin, whose absorption is aided by the dipeptide transporter exhibit very low diffusion through this cell line (Chong et al., 1996). Using both a cell culture and excised tissue model to study diuretic secretion, an increased likelihood of similar results *in vivo* can be expected.

Table 2-2: Strengths and weaknesses of the Caco-2 model.

Advantages	Disadvantages
• Resembles polarized enterocytes with well differentiated brush border.	• Resembles colon with low paracellular permeability.
• Well characterized at both molecular and functional levels.	• Lack Peyer's patches and mucous-secreting Goblet cells.
• Correlates well with human absorption for most compounds.	• Expression levels may be too high for efflux transporters, and too low for absorptive transporters.
• Contains most Phase II enzymes and carboxylesterases.	• Phase I enzymes low, or lacking.
	• Long culture times.

2.3 Key variables affecting transport across excised rat intestinal tissue

The conditions for studying intestinal transport across excised rat tissue are not well standardized despite the large number of researchers using this model. A few key variables are summarized in table 2-3. It is obvious that no clear choice for these variables has been reached by the various groups working with this system. Animal age and gender are often overlooked even though expression of P-gp has been shown to vary with "age" of cultured cells, and its expression and pattern of regulation have been found to be gender specific in studies using rat livers (Salphati and Benet, 1998). Some recorded conditions such as fasting of the animals, or not, prior to study are simply recorded with no explanation or preference anywhere in the literature. Removing muscle fibers to create, "stripped mucosa" removes the submucosal (and subvascular) tissue that many drugs would never encounter *in vivo* (Foulkes, 1996; Turner *et al.*, 1970), but use of "unstripped" tissue is nearly as common with the major advantages being greater ease and reduced likelihood of introducing holes. For our studies where transport in both directions was determined (Flanagan and Benet, 1999), any sink or increased barrier caused by underlying tissues would be expected to contribute equally regardless of transport direction and thus be irrelevant to the net secretion.

The buffers used for *in vitro* studies have long been known to affect transport and viability (Benet *et al.*, 1971) and the use of media developed to stabilize cultured cells has benefited excised tissue studies as well (Barthe *et al.*, 1998). Some variation exists in composition of buffers, but most researchers use a mixed salt solution at pH 7.4, as noted in table 2-3. The use of glucose and oxygenation is presumed, but not always noted. Glucose levels are highly variable in these studies ranging from 0.125 to 40 mM. The

Table 2-3: Variations in diffusion chamber methods over time.

Reference	Fasted	Stripped	Buffer[A]
Tai and Jackson, 1982 (male rat)	Yes	Yes	Buffer, pH=7.5 10 mM glucose (glc)
Grass and Sweetana, 1988 (male rabbit)	Yes	Yes	Krebs-Ringer's bicarbonate (KRB), 40 mM gl (S) or mannitol (M)
Yamashita *et al.*, 1990 (male rat)	?	No	Ringer's , 10 mM glc
Goerg *et al.*, 1992 (female rat)	No	Yes	Phosphate-bicarbonate, 12 mM glc
Artursson *et al.*, 1993 (female rat)	No	Yes	KRB
LeCluyse *et al.*, 1993 (rat)	No	Yes	Krebs-Henseleit
Pantzar *et al.*, 1994 (male rat)	No	No	KRB, 13.4mM glc
Yodoya *et al.*, 1994 (male rat)	Yes	Yes	KRB
Swaan and Tukker, 1995 (male rat)	No	Yes	TRIS-Ringer, 0.125 mM glc (S) or mannitol (M)
Lindley *et al.*, 1995 (male rat)	Yes	Yes	Ringer-bicarbonate, 10 mM glc (S) or mannitol (M)
Saitoh *et al.*, 1996 (male rat)	Yes	No	Tyrode's, 6 mM glc
Makhey *et al.*, 1998 (male rat)	Yes	Yes	pH 6.5 (M), 7.4 (S) Ringer's
Osman *et al.*, 1998 (rat)	No	No	KRB, 13.4 mM glc
Flanagan and Benet, 1999 (male rat)	No	No	KRB, 40 mM glc (S) or mannitol (M)

A. Buffers (name included if listed) were added to both serosal (S) and mucosal (M) sides and used at pH of 7.4 unless otherwise noted.

variable that could provide the greatest variation in studies between different labs is to which side of the membrane glucose is added.

The presence of intestinal nutrients such as glucose have been shown to increase solvent drag (Pappenheimer and Reiss, 1987) by direct opening of tight junctions (Sadowski and Meddings, 1993) in rat small intestine. Studies in humans have shown that even small amounts of glucose on the mucosal side can cause marked increases in accumulation of sodium and glucose on the serosal side (Fordtran *et al.*, 1965). Designers of a popular diffusion cell (Grass and Sweetana, 1988) have suggested that mannitol, a relatively non-absorbable sugar, be used on the mucosal side to prevent the rapid development of an osmotic gradient during studies, although this has seemingly been ignored by other researchers.

The potential for creating artifacts has not been well studied and few groups even measure transport in both directions, yet examples can be found. Diffusion of passively absorbed paracellular marker molecules, such as mannitol and inulin, which have been and still are presumed to be independent of direction, were found to be direction dependent when glucose was added to both sides. This prompted Pantzar and coworkers (1994) to conclude that the intestine is more permeable in the outward direction. Tai and Jackson (1982) also added glucose to both membrane surfaces and found similar directional diffusion for a series of weak bases, for which absorption has never been shown to be anything but passive. In our lab, mannitol is used on the mucosal side to maintain osmolarity. Using the methods described in Chapter 4 (Flanagan and Benet, 1999), we have shown no such outward preference for permeation of naproxen, a marker of passive trancellular absorption as shown in fig. 2-1. The effect of luminal glucose on *in vitro* transport has not been studied. It should be noted that all cell culture studies provide glucose on both sides of the membrane.

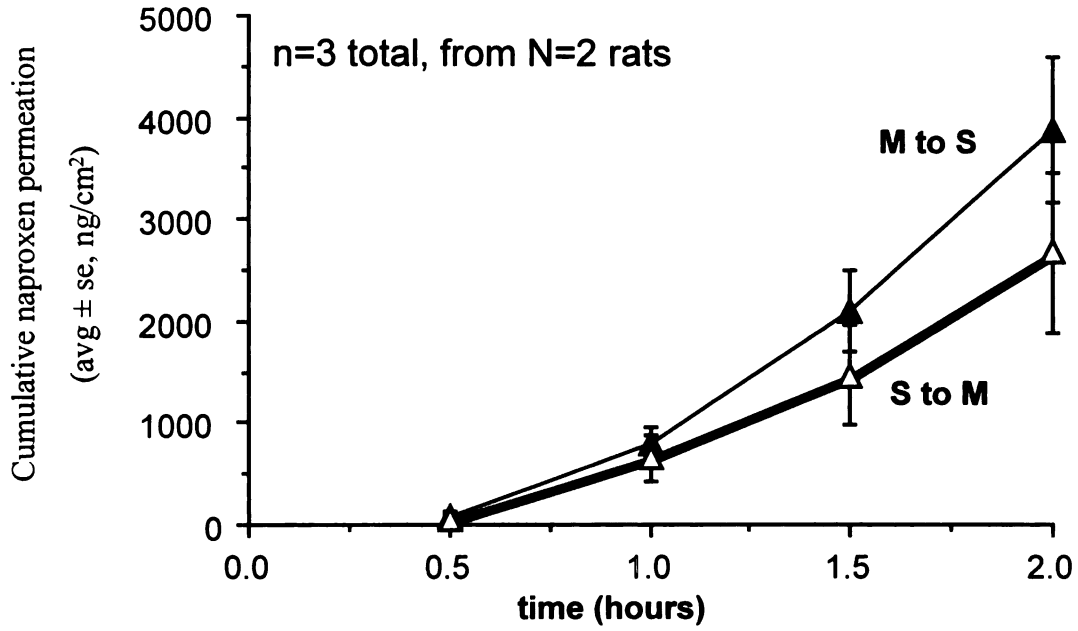


Figure 2-1: Directional permeation of naproxen (50 μM) across excised male Sprague-Dawley rat jejunum.

2.4 Key variables affecting transport across Caco-2 cells

Experiments using Caco-2 cell layers are more complicated and less standardized than those with excised rat intestine in terms of the variables involved. A recent review by Delie and Rubas (1997) reported permeability measurements by various researchers for thirteen molecules, including 8 drugs. Most of the compounds showed large variation between laboratories in the measured values, leading the authors to speculate that the different origin of the Caco-2 cells and culturing conditions had resulted in vastly different subclones in the hands of each of the different researchers. A confounding number of experimental conditions are being used to study transport and it is difficult to assess the influence of each. Some of the key variables thought to influence permeability

of various compounds are shown in table 2-4 and are summarized in the following sections.

Table 2-4: Variables that can affect Caco-2 transport.

Variable	Effect
Stirring	Absence can cause underestimate of permeation of some (rapidily absorbed) compounds.
Passage number	Transport proteins, such as P-gp and MRP, can vary.
Timing of splitting	If split after confluency, P-gp can be markedly reduced.
Timing of study	P-gp levels vary with age of cells.
Solvents	Can increase solubility, decrease activity (driving force), increase lipid bilayer fluidility, affect transporters and/or enzymes, and cause toxicity.

2.4.1 Membranes and culture equipment

There are a wide variety of permeable membranes available for diffusion studies. Polycarbonate (PC) membranes are available from Corning (Corning, NY) and Becton Dickinson, which also make polytetrafluoroethylene (PTFE) and polyethylene terephthalate (PET) membranes, respectively. In addition, both manufacturers also supply membranes coated for increased cellular attachment and promotion of differentiation. Fibrillar collagen coating (primarily from rat tail) has been used for Caco-2 cells and is required for both the 3-day BIEDE version of these cells and for cells induced to upregulate CYP3A4 (Schmiedlin-Ren *et al.*, 1997). Caco-2 cells grown on collagen coated membranes are ready for use about a week faster than those grown on uncoated filters, but some strange anomalies in growth have been reported by Hilgers *et al.* (1990) who observed the protrusion of cells into the pores of coated membranes. In addition, there is wide variation in available membrane pore sizes, ranging from 0.1 μm

to 12 μm openings. Their effect on cell growth and transport of drugs has not been studied.

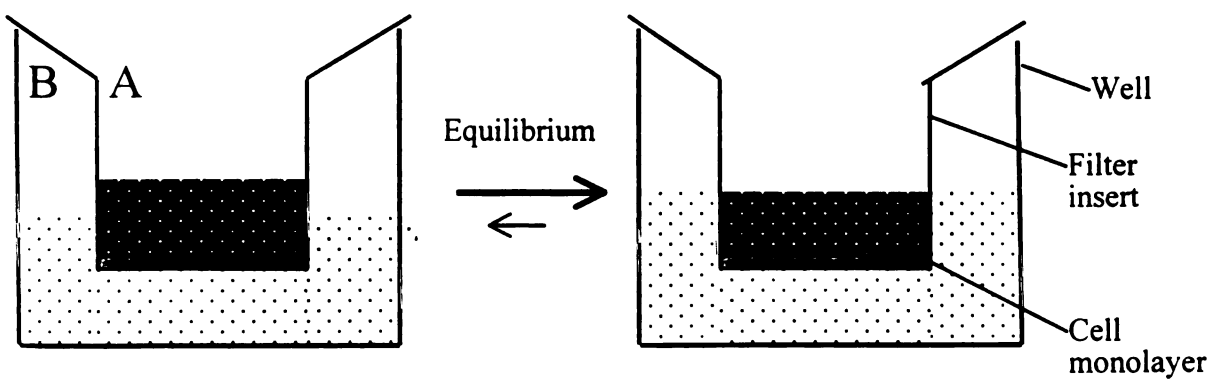
Cell monolayers grown on these inserts can be studied in a variety of different systems. Most are standard cell culture plates, but they vary in diffusive areas and volume ratios of basolateral and apical solutions depending on whether a 6-, 12- or 24-well plate is used as shown in table 2-5. The 24-well plate is more automation friendly, but requires high dexterity for manual operations and most researchers report using 6- or 12-well formats. Factors such as non-specific binding to plasticware, maintenance of boundary conditions, and effectiveness of stirring could vary tremendously based on architecture of these systems. Another important consideration is that the fluid levels in both sides should be equal when full. The exact amount needed to fulfill this requirement varies between manufacturers, but failure to do so could result in the creation of artifact-causing hydrostatic forces, as described in fig. 2-2.

The need for adequate stirring in diffusion testing is taken very seriously in most pharmaceutical testing, but has been overlooked in many Caco-2 transport studies. Karlsson and Artursson (1992) demonstrated using a form of the diffusion cells developed by Grass and Sweetana (1988) adapted to fit cultured cells, that permeability coefficients could be determined independent of the unstirred water layer. Their results showed that permeability of rapidly absorbed compounds, such as testosterone, were underestimated using standard 6-well plates and agitation mixing shakers. The benefit these new systems provide is contrasted by the increased labor requirements and decreased numbers of experiments that can be run in the same amount of time. Many studies report results without any mention of stirring.

Table 2-5: Diffusional surface area and loading volumes of membrane inserts.

Reference	Plate Format	Surface area (cm^2)*	Apical volume (ml)	Basolateral volume (ml)	Volume ratio B/A
Hunter *et al.*, 1993a	6-well	4.7	3.0	3.0	1.0
Tanaka *et al.*, 1995			1.5	2.6	1.7
Flanagan and Benet, 1999			1.5	2.5	1.7
Sakai *et al.*, 1994	12-well	1	2.5	2.5	1.0
Hosoya, *et al.*, 1996			0.4	1.5	3.8
Duizer *et al.*, 1998			0.5	1.8	3.6
Collett *et al.*, 1999			0.5	1.5	3.0
B-D recomendation	24-well	0.3	0.3	1.0	3.0

* Data shown for Corning, varies slightly between manufacturers.



2.0 ml Apical and 2.0 ml Basolateral

1.5 ml Apical and 2.5 ml Basolateral

Figure 2-2: Possible artifacts related to loading volume.

2.4.2 Culturing conditions

Since Caco-2 cells are polymorphic in nature, the way that they are maintained can change them over time. Choice of growth media, feeding schedule, time of last feeding, and total days in culture (post-seeding vs. post-confluence) could result in selection of subcultures or markedly alter expression levels and transport properties of these cells. Several researchers (e.g. Hosoya *et al.*, 1996) found that P-gp levels start out high (after one week), then decrease and slowly build up again so that four week measurements approximate the one week values. The protein may not be functional at the early age. Variations in expression levels may be part of the differentiation process that occurs after reaching confluency. So if the seeding density (which more or less controls time to confluency under identical conditions) is not fairly constant for a given researcher, then studies should be run at a defined period post-confluence.

The issue of confluency at the time the cells are split can be an important variable in itself and have a marked effect on the expression levels of transport proteins such as P-gp. Anderle *et al.* (1998) found that if cells were split prior to 70% confluency for a series of passages, then P-gp levels would increase, whereas P-gp would decrease if the split occurred after confluency. Passage number is another source of variation. Makhey and colleagues (1998) found that expression levels of P-gp, MRP and LRP were highest at passage 47 and 63 and lower at both earlier (27) and later (92) passages.

2.4.3 Study conditions

The way that transport studies are conducted may influence the outcome. Studies are often conducted using the same media used for growth (possibly without additional supplements such as fetal bovine serum) unless the media interferes with the method of analysis. Studies using radiolabeled compounds often will use media that contains human serum albumin (Artursson and Magnusson, 1990) or even fetal bovine serum (an

essential component for growth), but when methods such as HPLC are required for analysis, a serum free buffer (Boulenc *et al.*, 1993) is most often used. Studies conducted with a radiolabeled compound in one media may not be equivalent to studies using LC-MS to detect the same compound in a different, cleaner buffer, because the cells may behave very differently under the different conditions. Permeants and inhibitors are usually dissolved in organic solvents, such as ethanol or DMSO, which can alter physiochemical parameters. Vehicle effects on viability, solubility and partitioning can have a marked influence on permeation independent of any transporter action. Care should be taken to ensure that changes seen as a function of concentration are actually due to the proposed mechanism and not merely a function of increasing solvent concentration. It is known that pH can have a marked influence on the transport of weak acids and bases across biological membranes, yet many researchers fail to demonstrate that pH is maintained even at high drug or inhibitor levels and a few do not even report these values.

2.5 Conclusions

A number of *in vitro* models have been employed to predict oral bioavailability. These models include the older use of excised tissue and the relatively newer use of cultured cells. There are advantages and disadvantages for each model, so a thorough understanding of the limitations is needed prior to use. We selected two models, Caco-2 cells and rat jejunal segments mounted on diffusion chambers, for our studies to better determine relevant transport properties of diuretics *in vitro*.

Chapter 3

Apparent Secretion of Amiloride in Excised Rat Jejunum and Cultured Adenocarcinoma (Caco-2) Cells

3.1 Introduction

Amiloride is an old drug used alone as a mild diuretic, or to limit the potassium loss associated with the more potent thiazide and loop diuretics, thus avoiding their major toxicity (hypokalemia). Few studies have been conducted on its pharmacokinetics and most of those were prior to 1974. Bioavailability is low and varies between reports from as low as 15 to 25% (Jackson, 1996) to as high as 50% or greater (Macfie *et al.*, 1981).

Recent studies in our laboratory and others have shown that the small intestine functions as a major organ of elimination for orally administered drugs (Mayer *et al.*, 1996; Paine *et al.*, 1996; Wu *et al.*, 1995). Efflux transporters, such as P-glycoprotein (Thiebaut *et al*, 1987; Watkins, 1997), and cytochrome P450 drug-metabolizing enzymes have been shown to be localized at the tips of intestinal microvilli where they are believed to serve a primarily defensive role in preventing xenobiotics from entering the general circulation (Watkins, 1997).

Active secretion of amiloride is carried out by renal transporters of the organic cation transport system in the proximal tubule of the nephron (Besseghir and Rennick, 1981), a mechanism by which the drug reaches its site of action in the collecting tubule (Ives and Warnock, 1995). As similar drug transporters are found in the kidney and the intestine, secretion in the small intestine would be expected to reduce bioavailability. This study focuses on the question of whether intestinal secretion of amiloride occurs *in vitro* in two models of intestinal absorption.

3.2 Materials and methods

3.2.1 Materials

The Caco-2 cell line (HTB-37), purchased from the American Type Culture Collection (ATCC, Rockville, MD), and all media were obtained from the UCSF Cell Culture Facility (San Francisco, CA). Male Sprague-Dawley rats were purchased from Charles River Laboratories (Willmington, MA). Cyclosporine was a gift from Sandoz Pharmaceuticals (Basel, Switzerland). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

3.2.2 Preparation of the isolated sheets of rat jejunum and transport studies

Studies were approved by the UCSF committee on Animal Research. Rats were allowed free access to food and water prior to sacrifice. The gut was then exposed surgically and a cut was made in the small intestine at the junction between the duodenum and jejunum. Another cut was made somewhere in the vicinity of the ileum (approximately 20 cm distal to the first cut) and this section of small intestine was removed. The intestinal section was washed with 20 ml of ice cold Krebs-Ringer Bicarbonate solution (KRB),

and cut into six segments about 3 cm in length, starting at the most proximal end. Peyer's patches were avoided and the segments were placed in numbered petri dishes filled with ice cold buffer, such that the highest numbers were segments closest to the ileum. The numbered segments were randomly assigned to each of the test formulations to avoid any variation due to location.

Tweezers were used to remove fat from each segment prior to cutting with scissors along the mesentarium and the resulting flat sheet was mounted in modified Ussing type chambers (Navicyte, Sparks, NV). The serosal (i.e., basolateral) and mucosal (i.e., apical or brush border) sides were filled with 6 ml of warm KRBS containing 40 mM glucose or 40 mM mannitol respectively, and allowed to equilibrate at 37 °C for 30 min. Prior to starting the study, a small volume was removed from the donor and receiver sides and replaced with equal volumes of permeant from concentrated solutions, such that the starting volume remained 6.0 ml. Amiloride (20 μM from a 1.2 mM stock solution in KRBS) was added to the donor side. All studies were conducted at a pH of approximately 7. Samples were collected in 500 μl aliquots every 30 min for 2 hr, followed by replacement with mucosal or serosal buffer. Studies were conducted in both the mucosal-to-serosal, and serosal-to-mucosal directions over a surface area of 1.78 cm^2.

3.2.3 Preparation of cell culture monolayers and transport studies

All cells were cultured at 37 °C and humidified, 5% CO_2-atmosphere, in medium containing 10% fetal bovine serum. Minimum Essential Medium (MEM) Eagle's with 2 mM L-glutamine and Earle's BSS containing 1.5 g/L sodium bicarbonate, was also supplemented with 0.1 mM non-essential amino acids and 1.0 mM sodium pyruvate. Caco-2 cells grown to confluence (passages 25-35) in culture flasks were harvested with 0.05% Trypsin EDTA and seeded onto polycarbonate filters (0.4 μm pore size, Fisher, Pittsburgh, PA) in 6-well cluster plates at an approximate density of 10^6 cells/insert.

Studies were conducted at 21 days post seeding. Media was changed at least twice per week, including 18 to 24 hr prior to testing.

Studies were conducted after equilibrating the cells at 37 °C for 30 min in fresh media without supplements. Testing was done with apical and basolateral chamber volumes of 1.5 and 2.5 ml, respectively. Both the basolateral-to-apical and apical-to-basolateral directions were tested over a surface area of 4.71 cm^2. Amiloride and inhibitors (added to both sides) were added from concentrated DMSO solutions for a final solvent concentration in the transport media of exactly 1% and pH of approximately 7.4, and studies were conducted at 37°C (incubator shaker), unless otherwise noted. Integrity of monolayers was assessed by measuring transepithelial electrical resistance (TEER) using a Millipore Millicell-ERS resistance system (Millipore Corporation, Bedford, MA). Average TEER values for the monolayers were approximately 400 Ohms/4.7 cm^2. Sampling was done without replacement at 1 and 1.5 hr (100 µl and 50 µl, respectively), from the receiver and the entire solution was removed at 2 hr. The data in each figure or table refers to an individual experiment with its own control from a single batch (same source vial) of cells.

3.2.4 Analytical methods

Samples in media or buffer were analyzed by reverse-phase chromatography and fluorescent detection without extraction. Briefly, amiloride analysis was performed with a 4.6 mm x 25 cm Ultrasphere Silica column, 7.5 mM ammonium phosphate, dibasic and 60% acetonitrile isocratic mobile phase, with excitation and emission wavelengths of 355 and 410 nm, respectively.

3.3 Results

3.3.1 Bidirectional transport of amiloride

Diffusion studies were conducted using excised male Sprague-Dawley rat jejunum mounted on modified Ussing chambers. This *in vitro* system is well accepted as a model for human intestine and has been used to demonstrate intestinal secretion (Saitoh and Aungst, 1995). Amiloride was found to accumulate preferentially on the mucosal, i.e. apical, side of the membrane (fig. 3-1). The total amount of amiloride that accumulated on the mucosal side in two hr was more than 4-times as much as that accumulated on the serosal side (fig. 3-1).

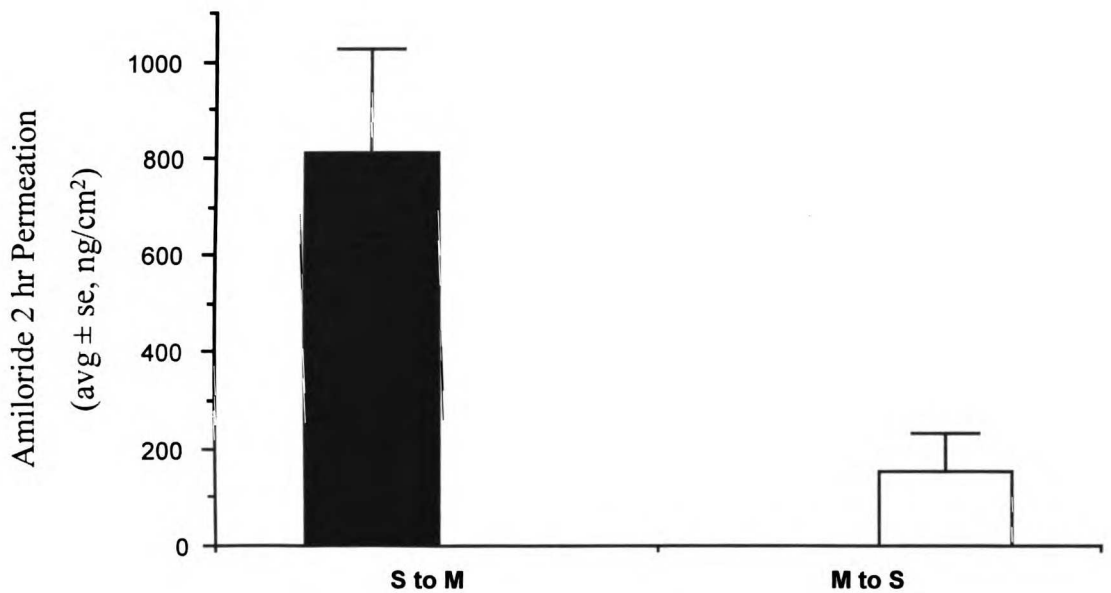


Figure 3-1: Total permeation of amiloride (50 μM) through excised male Sprague-Dawley rat jejunum at 2 hr.

Another system commonly used to study intestinal absorption involves the use of Caco-2 cells grown on polycarbonate filter inserts (see critical review by Delie and Rubas, 1997). Diffusion studies of amiloride transport across Caco-2 cells confirmed the rat jejunum

results, showing net secretion (figure 3-2). Small net secretion of amiloride was always observed using Caco-2 cells, but significant differences (* $p<0.05$) were only observed in half of the studies. Overall magnitude varied between 1.4- and 3-fold and on average was less than half of that seen with rat jejunum. Total permeation varied considerably within Caco-2 experiments as can be seen from subsequent figures and was lower than for rat jejunum.

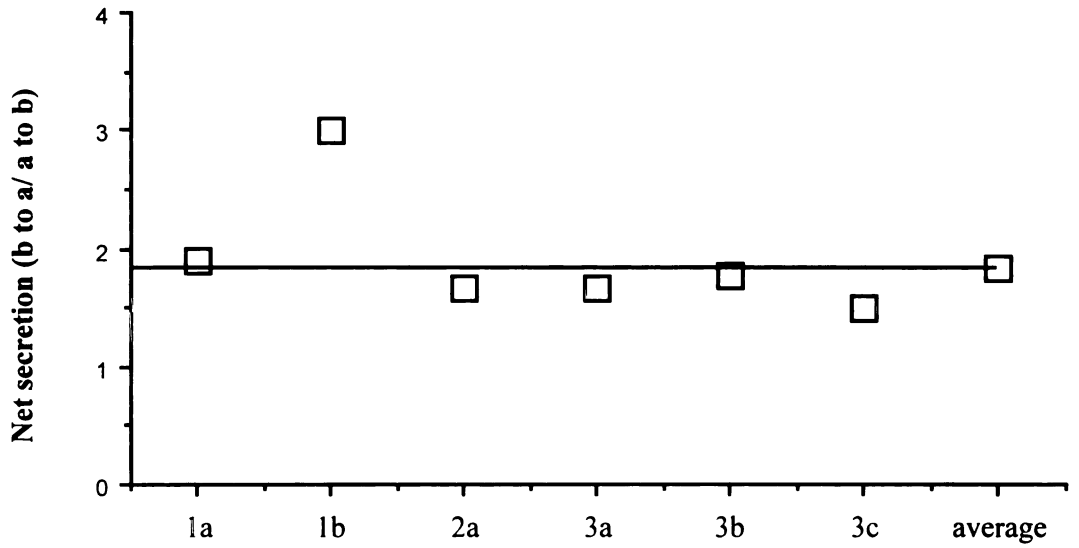
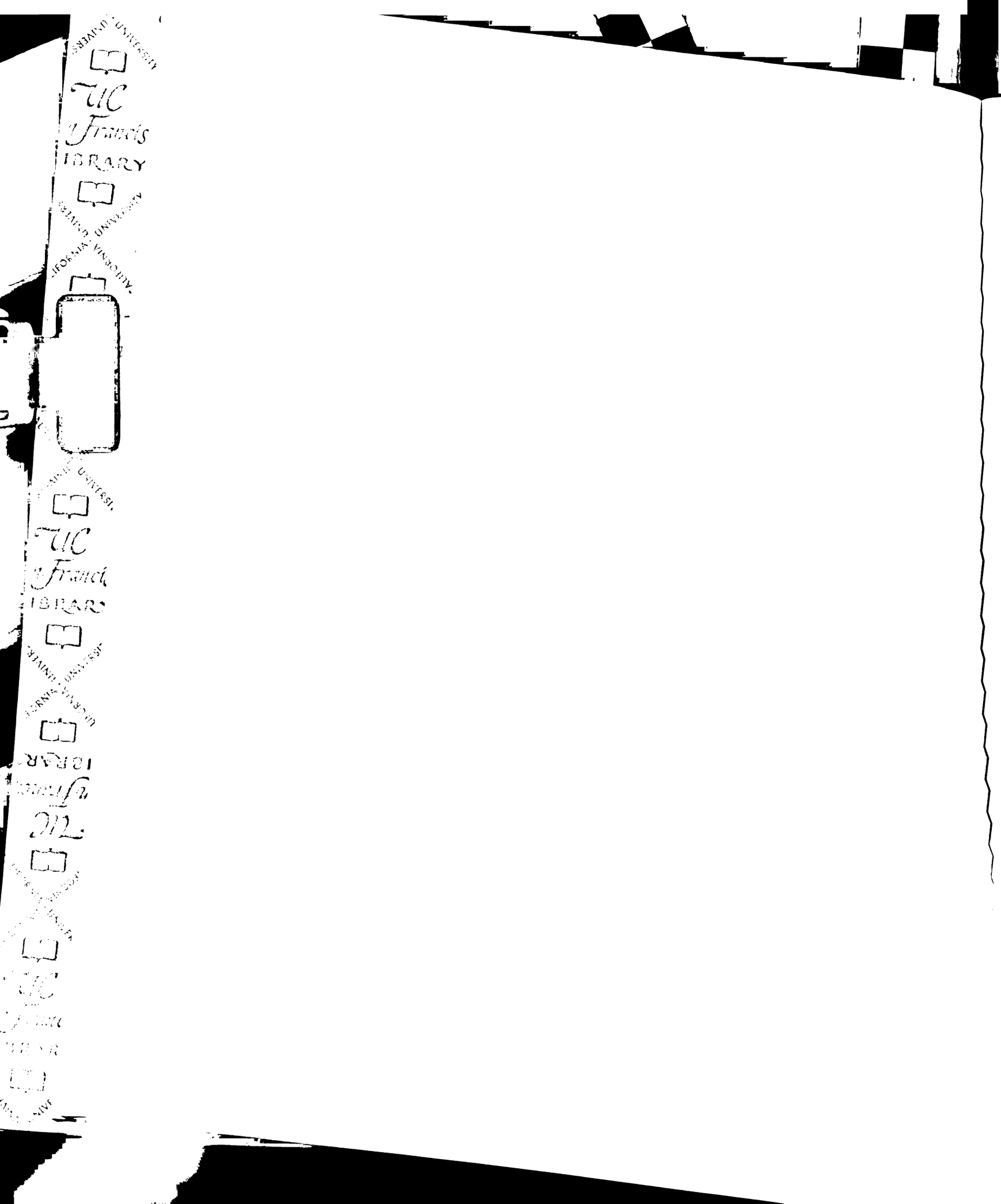


Figure 3-2: Net secretion of amiloride (20 μM) by Caco-2 cells, at 2 hrs, was observed in six separate studies (each with n=3 in both B to A and A to B directions) from three separate source vials of cells (1, 2 and 3). Suffixes a, b and c refer to the first , second and third passages, respectively, after thawing of source cells. Mean net secretion is indicated by the solid line.

3.3.2 Effect of concentration and temperature on transport of amiloride

Figure 3-3 shows the effect of temperature on the net secretion of amiloride by Caco-2 cells. In this study, transport was reduced in both the B to A (22%) and A to B (36%) directions when the temperature was reduced from 37 to 4°C. These changes were not



significantly different are relatively minor compared to temperature-induced decreases associated with carrier-mediated processes.

Amiloride permeation through Caco-2 cells grown on filter inserts was measured over a concentration range of 5 to 200 μM. Permeation was found to be linear with time at each concentration tested (data not shown) and although greater in the B to A direction at all concentrations, no saturation was seen (fig. 3-4 insert). Both A to B and B to A data could be fit to a linear equation with r^2 values of 0.998 and 0.997, respectively. Since transport was linear, concentrations were increased to 2 mM (which is approximately 80% of solubility) in a separate study (fig. 3-4). Fluxes of amiloride over the range of 20 to 2000 μM were also linear with concentration.

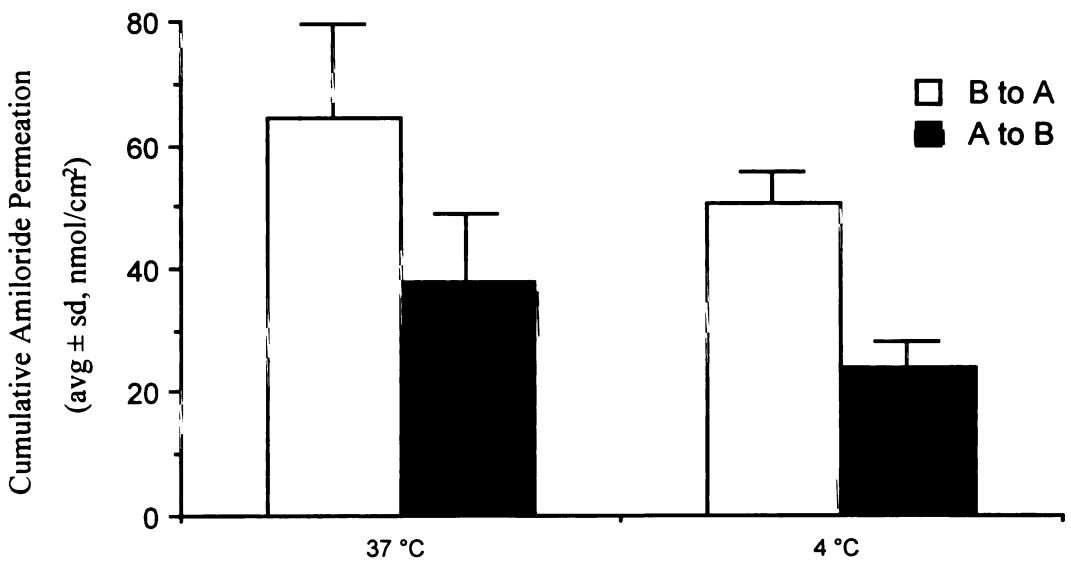


Figure 3-3: Effect of temperature on amiloride (20 μM) permeation through Caco-2 epithelia at 2 hr. Decreased temperature did not cause a significant reduction in directional peremation.

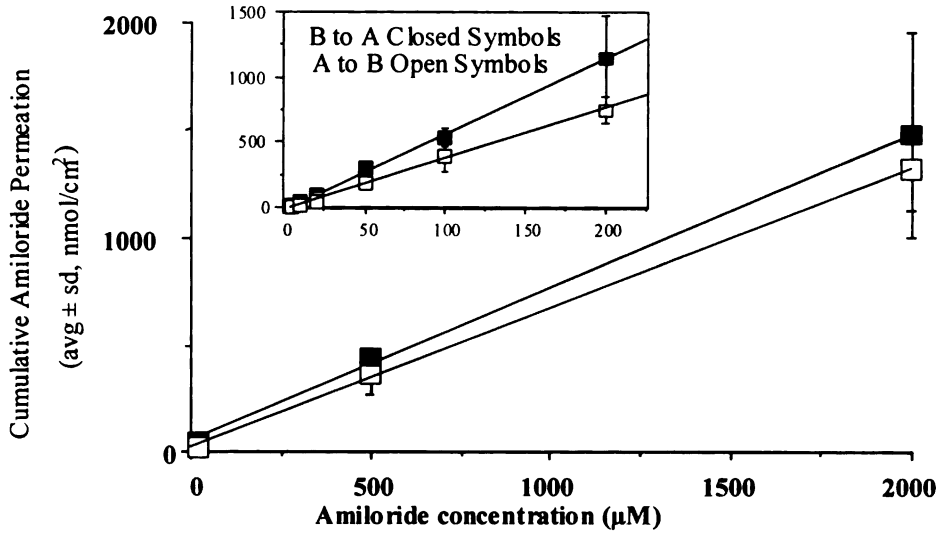


Figure 3-4: Amiloride concentration vs. permeation through Caco-2 epithelia at 2 hr. Insert shows data from a separate experiment.

3.3.3 Effect of inhibitors on transport of amiloride

Amiloride permeation across Caco-2 cells was tested for sensitivity to inhibition. Tetraethylammonium (TEA) a known inhibitor of OCT, and cyclosporine, an inhibitor both of MRP and P-gp, were tested for their effect on the directional transport of amiloride. No effect on amiloride secretion was observed (fig. 3-5). Cyclosporine (CsA) was tested near the limit of solubility and could not be increased, but increasing TEA concentrations up to 2 mM had no affect on amiloride transport (data not shown).

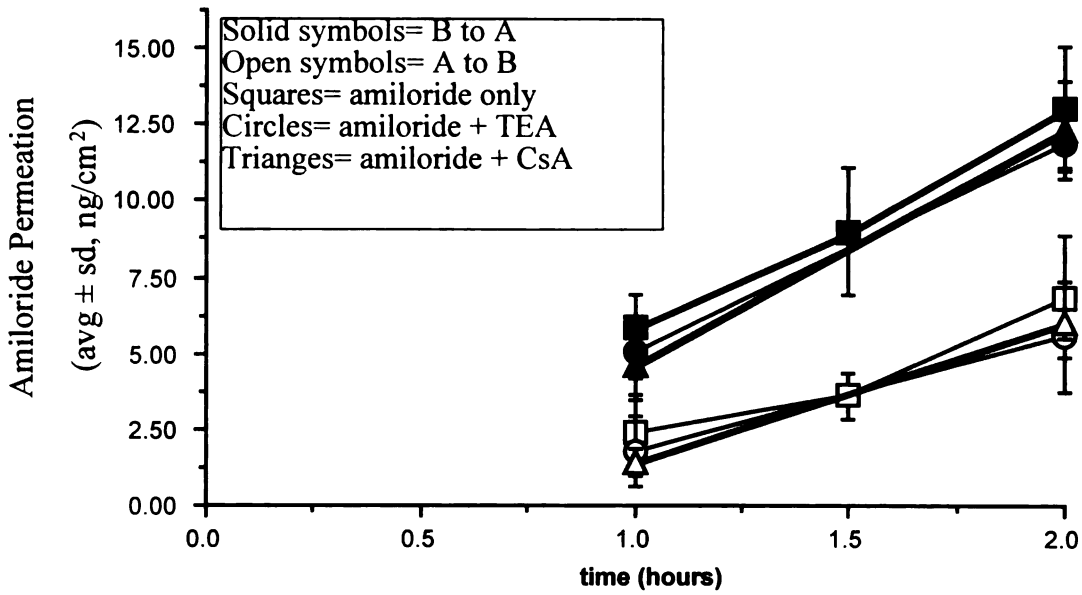


Figure 3-5: Effect of potential inhibitors TEA (100 μM) and CsA (50 μM) on permeation of amiloride (20 μM) through Caco-2 epithelia.

3.4 Discussion

Amiloride transport across excised rat jejunum and cultured Caco-2 cells was found to be greater in the direction of secretion (figs. 3-1 and 3-2). This is consistent with our hypothesis that this compound may be actively secreted in the small intestine causing its poor bioavailability. The differences between experiments in terms of absolute magnitude of amiloride permeation are hard to explain. Comparison of B to A permeation from 20 μM starting concentrations within Caco-2 experiments showed a range of nearly 5-fold (fig. 3-3 vs. 3-5). A similar difference was observed when comparing B to A permeation from 50 μM starting concentrations between rat jejunum and Caco-2 experiments (fig. 3-1 vs. 3-4A). If amiloride has a large paracellular component to its intestinal permeation, then it would be expected to permeate across

Caco-2 cells to a lesser extent than the rat tissue, given that Caco-2 cells have tighter junctions than rat (and human) small intestine. Differences between Caco-2 experiments where initial TEER measurements where similar is difficult to rationalize.

Two features of carrier-mediated transport, temperature and concentration dependence, were tested in order to characterize the transport of amiloride. A small, insignificant, reduction in directional permeation was seen for amiloride as the temperature was decreased from 37 to 4 °C, but the net secretion actually went up from 1.7- to 2.1 fold (fig. 3-3) indicating that a carrier protein is unlikely to be involved. Similarly, the directional transport did not display saturable dependence on concentration over the tested range of concentrations (fig. 3-4). The possibility that we are still in the linear range cannot be tested as amiloride is close to its limit of solubility at the highest concentration. Likewise we cannot rule out the possibility that we have greatly exceeded the K_m even at the lowest concentrations and are only seeing the dominant passive component of permeation because of limits of detection.

Studies using TEA and cyclosporine failed to produce inhibition (fig. 3-5), providing additional evidence against the involvement of transporters such as OCT, MRP or P-gp. Comparison of amiloride permeation across MDCK and MDR1-MDCK cells also failed to implicate P-gp (data not shown).

Tai and Jackson (1982) reported that there is a directional difference for transport of weak bases across rat intestine. These directional differences could be the result of an efflux transporter such as P-gp. Differences in directional permeation intrinsic to the intestine may better explain the observed amiloride results. Paracellular transport can be affected by drugs such as verapamil (Sakai *et al.*, 1994) and ranitidine (Gan *et al.*, 1998). Perhaps, amiloride has an affect on its own paracellular transport that results in directional

differences. If high concentrations on the basolateral side are required to open tight junctions, then the result would resemble secretion, as would high apical concentrations causing a closing of tight junctions. Our results indicate that amiloride's apparent secretion is unlikely to be caused by a transporter, and may be due to either intrinsic differences in directional permeation or an affect on paraceullar transport. Thus, the proposed hypothesis that intestinal secretion causes low bioavailability of amiloride appears to be incorrect.

Chapter 4

Net Secretion of Furosemide Is Subject to Indomethacin Inhibition, as Observed in Caco-2 Monolayers and Excised Rat Jejunum*

4.1 Introduction

Furosemide is a potent and widely used diuretic with highly variable and poor bioavailability, 11 to 90% (Jackson, 1996). The pharmacokinetics and pharmacodynamics of this drug have been extensively studied and reviewed (Hammarlund-Udenaes and Benet, 1989), yet the cause of this low and variable bioavailability remains unclear. Bioavailability from oral solution and tablet formulations are not different, suggesting that solubility and dissolution rates are not important factors (Kelly *et al.*, 1974). Gastric elimination by acid hydrolysis has also been ruled out as a significant cause for the drug's poor bioavailability (Bundgaard *et al.*, 1988). A possible explanation is that furosemide may be secreted by the intestine. This has been widely speculated (Lee and Chiou, 1983; Valentine *et al.*, 1986; Verbeeck *et al.*, 1982), but never demonstrated.

In 1986, Valentine and co-workers showed that half of the elimination of an intravenous furosemide dose occurred non-renally and non-metabolically, but that experimentally

* This chapter is slightly revised from a recent publication (Flanagan and Benet, 1999).

active secretion into the gut could not be demonstrated. Studies in our laboratory suggested that significant metabolic "non-renal" elimination of furosemide occurs by glucuronidation in the kidneys (Smith and Benet, 1983). A recent study, using isolated rabbit kidneys has demonstrated this hypothesis to be valid (Pichette and du Soich, 1996). We believe that secretion of orally administered furosemide could be a significant factor in limiting bioavailability. Intestinal elimination of drugs is expected to be higher for orally administered drugs than for systemically administered compounds due to liver and intestine first-pass effects, as has been observed for midazolam in transplant patients (Paine *et al.*, 1996) and for digoxin in mice (Mayer *et al.*, 1996).

Recent studies in our laboratory and others have shown that the small intestine can function as a major organ of elimination (Mayer *et al.*, 1996; Paine *et al.*, 1996; Wu *et al.*, 1995). Efflux transporters, such as P-glycoprotein (Thiebaut *et al.*, 1987; Watkins, 1997), and cytochrome P450 drug-metabolizing enzymes are localized at the tips of intestinal microvilli where they are believed to serve a primarily defensive role in preventing xenobiotics from entering the general circulation (Watkins, 1997).

Active secretion of furosemide is carried out by renal transporters of the organic anion transport system in the proximal tubule of the nephron, a mechanism by which the drug reaches its site of action in the lumen of the loop of Henle (Jackson, 1996). As similar drug transporters are found in the kidney and the intestine, secretion in the small intestine would be expected to reduce bioavailability. This study focuses on the question of whether intestinal secretion of furosemide occurs in two *in vitro* models of intestinal absorption, and provides preliminary information for future mechanistic studies.

4.2 Materials and methods

4.2.1 Materials

The Caco-2 cell line (HTB-37), purchased from the American Type Culture Collection (ATCC, Rockville, MD), and all media were obtained from the UCSF Cell Culture Facility (San Francisco, CA). Male Sprague-Dawley rats were purchased from Charles River Laboratories (Willmington, MA). Cyclosporine was a gift from Sandoz Pharmaceuticals (Basel, Switzerland). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

4.2.2 Preparation of the isolated sheets of rat jejunum and transport studies

Rats were allowed free access to food and water prior to sacrifice. A cut was made in the small intestine at the junction between the duodenum and jejunum, and a section of small intestine (approximately 20 cm) was removed. The intestinal section was washed with 20 ml of ice cold Krebs-Ringer Bicarbonate solution (KRB), and cut into six segments about 3 cm in length, starting at the most proximal end. Peyer's patches were avoided and the segments were placed in numbered petri dishes filled with ice cold buffer, such that the highest numbers were segments closest to the ileum. The numbered segments were randomly assigned to each of the test formulations to avoid any variation due to location.

Tweezers were used to remove fat from each segment prior to cutting with scissors along the mesentarium and the resulting flat sheet was mounted in modified Ussing type chambers (Navicyte, Sparks, NV). The serosal (i.e., basolateral) and mucosal (i.e., apical or brush border) sides were filled with 6 ml of warm KRBS containing 40 mM glucose or 40 mM mannitol respectively, and allowed to equilibrate at 37 °C for 30 min. Prior to

starting the study, a small volume was removed from the donor and receiver sides and replaced with equal volumes of permeant from concentrated solutions, such that the starting volume remained 6.0 ml. Furosemide (20 μM from a 1.2 mM stock solution in KRBS) was added to the donor side, and for the inhibition studies indomethacin (100 μM from a 40 mM stock solution in DMSO) was added to both sides. All studies were conducted with 0.25% DMSO, and a pH of 6.5. Samples were collected in 500 μl aliquots every 30 min for 2 hr, followed by replacement with mucosal or serosal buffer. Studies were conducted in both the mucosal-to-serosal, and serosal-to-mucosal directions over a surface area of 1.78 cm^2.

4.2.3 Preparation of Caco-2 monolayers and transport studies

Caco-2 cells (25-35 passages) were cultured at 37 °C and humidified, 5% CO_2-atmosphere, in Minimum Essential Medium (MEM) Eagle's with 2 mM L-glutamine and Earle's BSS containing 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, and 10% fetal bovine serum. Cells grown to confluence in culture flasks were harvested with 0.25% Trypsin EDTA and seeded onto polycarbonate filters (0.4 μm pore size, Fisher, Pittsburgh, PA) in 6-well cluster plates at an approximate density of 10^6 cells/insert. Studies were conducted at 21 days post seeding. Media was changed at least twice per week, including 18 to 24 hr prior to testing.

Studies were conducted after equilibrating the cells at 37 °C for 30 min in fresh MEM without supplements. Testing was done with apical and basolateral chamber volumes of 1.5 and 2.5 ml, respectively. Both the basolateral-to-apical and apical-to-basolateral directions were tested over a surface area of 4.71 cm^2. Furosemide and indomethacin

(added to both sides) were added from concentrated DMSO solutions for a final solvent concentration in the transport media of 0.5 to 1% and a pH of 7.4. Integrity of monolayers were assessed by measuring transepithelial electrical resistance (TEER) using a Millipore Millicell-ERS resistance system (Millipore Corporation, Bedford, MA). Average TEER values for the Caco-2 monolayers were approximately 400 Ohms/4.7 cm^2. Sampling was done without replacement at 1 and 1.5 hr (100 µl and 50 µl, respectively), from the receiver and the entire solution was removed at 2 hr.

4.2.4 Analytical methods

Samples in media or buffer were analyzed by reverse-phase chromatography and fluorescent detection using previously published methods (Smith *et al.,* 1980a) without extraction. Briefly, furosemide analysis was performed with a 4.6 mm x 25 mm C 18 column, 0.1% phosphoric acid in 35% acetonitrile isocratic mobile phase, with excitation and emission wavelengths of 345 and 405 nm, respectively.

4.3 Results

Diffusion studies were conducted using excised male Sprague-Dawley rat jejunum mounted on modified Ussing chambers. This *in vitro* system has been used to demonstrate the secretion of the known P-gp substrate verapamil (Saitoh and Aungst, 1995), and we were able to reproduce these results with our systems (data not shown). Furosemide also appeared to be secreted, and was found to accumulate preferentially on the mucosal, i.e. apical, side (fig. 4-1). The total amount of furosemide that accumulated on the mucosal side in two hr was more than 3-times as much as that accumulated on the

serosal side (fig. 4-1). This result was repeated in a separate study in which the absolute permeation of furosemide in both directions was approximately one-third lower (not shown). Serosal-to-mucosal transport was substantially reduced by more than 200% with the addition of the weak organic acid indomethacin to both sides of the membrane, but the expected increase in mucosal-to-serosal transport was not seen and neither result was significantly different from the control due to large variations.

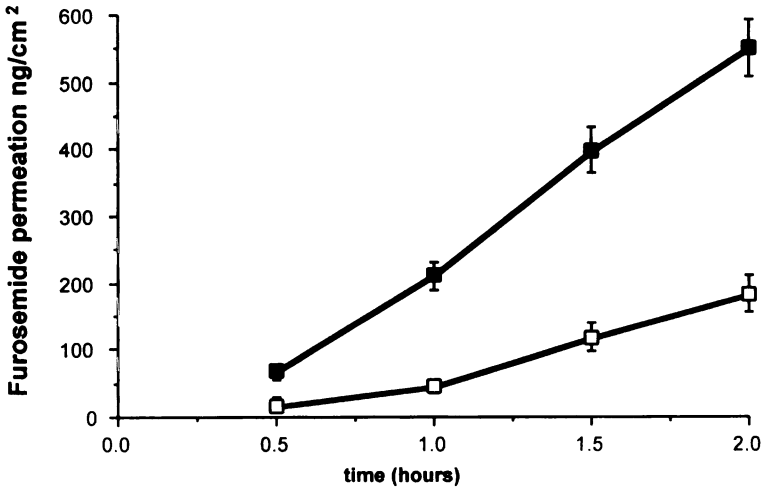


Figure 4-1: Permeation of furosemide (20 μM) through excised male Sprague-Dawley rat jejunum. Open squares for mucosal-to-serosal, and closed squares for serosal-to-mucosal directions. Each point represents the mean ± SE of n=10, from N= 6 rats.

Another system commonly used to study intestinal absorption involves the use of Caco-2 cells grown on polycarbonate filter inserts. Several laboratories have characterized

transport properties for this cell line and demonstrated it to be a highly useful model for absorption studies (Hidalgo *et al.*, 1989; Hilgers *et al.*, 1990) having few limitations (Delie and Rubas, 1997). Diffusion studies of furosemide transport across Caco-2 cells confirmed the rat jejunum results, showing greater accumulation on the apical side at 37°C (fig. 4-2). The net secretion (i.e., the total amount transported into the apical compartment in 2 hr divided by the total amount transported into the basolateral compartment, B-to-A/A-to-B) observed using this model was over 300% greater than for intestinal segments (10-fold *vs.* 3-fold, as depicted in figs. 4-2 and 4-1, respectively). This difference between the two models was the result of a greater decrease in total furosemide transport in the absorption direction than in the secretion direction.

Net secretion of furosemide by Caco-2 cells was reduced to less than 2-fold by decreasing temperature from 37° to 4°C (fig. 4-2), as the result of a nearly 85% decrease in B-to-A transport, with a less than 25% decrease in A-to-B transport. Transport in the A-to-B direction was not significantly different at 37° or 4°C.

Figure 4-2 also shows that indomethacin caused both significantly ($p < 0.05$) reduced B-to-A transport, and increased A-to-B transport. The resulting furosemide transport in the presence of indomethacin was no longer dependent on direction and thus resembled a purely passive process. Probenecid, cyclosporine, and vinblastine were also found to significantly reduce the secretion of furosemide in the B-to-A direction by 21 to 26%, as shown in table 4-1, but there was no effect on A-to-B transport for these compounds.

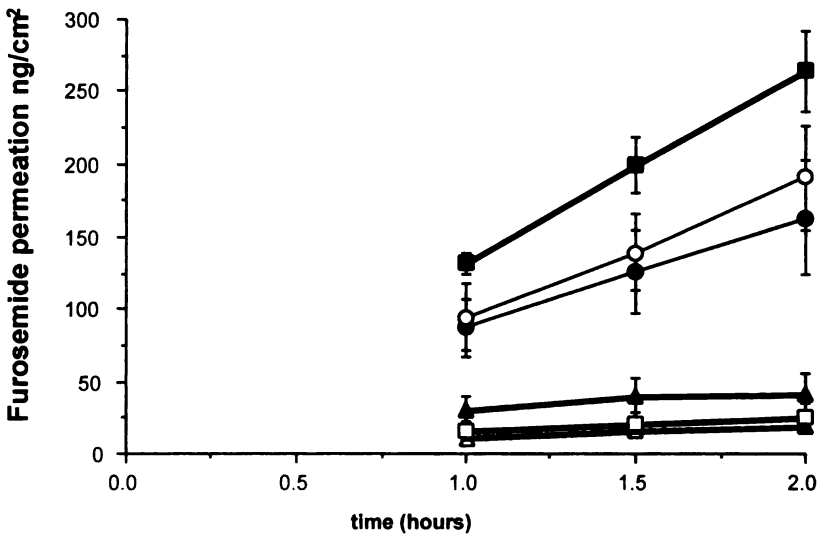


Figure 4-2: Permeation of furosemide (30 μM) through Caco-2 epithelia is subject to temperature and chemical inhibition. Open symbols for apical-to-basolateral, and closed for basolateral-to-apical directions. Controls (37 °C) are shown as squares, with triangles for reduced temperature (4 °C). Circles are used for indomethacin-treated (50 μM, 37 °C). Each point represents the mean ± SD from n=3.

Table 4-1: Inhibition of 30 μM Furosemide Transport From Caco-2 Cells

Inhibitor	Concentration	Percent decrease in B-to-A secretion
Cyclosporine	50 μM	21
Probenecid	200 μM	26
Vinblastine sulphate	40 μM	24

4.4 Discussion

Fick's First Law, as it applies to intestinal transport, tells us that the steady-state rate of transport, or flux, only depends on the concentration gradient across the membrane, and should be equal in both directions. This was not the case for furosemide whose transport showed a directional dependence across both excised rat intestine and cultured human intestine (Caco-2). This directional difference is unlikely to be the result of an artifact, as quantitation was done by a specific assay, and positive and negative controls for these transport systems have been well established within this laboratory. The fact that decreasing temperature from 37° to 4°C resulted in a substantial drop in net secretion suggests the involvement of a protein transporter and a carrier-mediated process. In addition, recent studies in our laboratory (see Chapter 5) have shown that secretion of furosemide is also saturable.

The apparent secretion of furosemide could be the result of an uptake or secretory transporter in the basolateral or apical intestinal membrane, respectively, or some combination. The inhibition of furosemide secretion seen with indomethacin treatment (fig. 4-2) provides further evidence of an active process in the intestine, but does not differentiate between these two transport processes. Indomethacin has long been known to inhibit renal clearance of many anionic xenobiotics including furosemide (Smith *et al.*, 1979), presumably through competition for kidney organic anion transporters (OAT). The prototype OAT substrate (*p*-aminohippurate) has been shown to be actively taken into kidney cells on both membranes, as well as effluxed across the apical membrane (Bendayan, 1996). It is not clear at this time if any of these transporters are present in the small intestine in a polarized manner that would result in net secretion, so it seemed

logical to perform the functional inhibition studies in intestinal cells to see if a similar process to that in the kidney occurred.

Indomethacin-sensitive inhibition of intestinal secretion has been observed for bis carboxyethyl carboxyfluorescein in several cell lines including Caco-2 (Collington *et al.*, 1992), and phenol red in excised rat jejunum (Saitoh *et al.*, 1996). These authors were unable to implicate a specific transporter, although Collington *et al.* (1992) speculated that "efflux may be mediated by an indomethacin-sensitive ATP-binding cassette transporter protein". The Multidrug Resistance-associated Protein (MRP) transporter is an ATP-binding cassette efflux transporter with weak homology to p-glycoprotein, capable of transporting many anions. Indomethacin is known to inhibit and thereby reverse multidrug resistance in human and murine cell lines expressing MRP (Draper *et al.*, 1997).

At least six different MRP's have been identified, but only MRP1 has been demonstrated to convey multidrug resistance (Kool *et al.*, 1997). In addition to indomethacin sensitivity, MRP1 has been shown to be inhibited by probenecid, vinblastine, and cyclosporine (whose effects on the intestinal secretion of furosemide are shown in table 4-1) in cancer lines transfected with or overexpressing MRP, but not p-glycoprotein (Holló *et al.*, 1996).

MRP-1 has been found in the small intestine, but has not been localized to a particular membrane. In transfected porcine kidney epithelial cells (LLC-PK1), it is found exclusively on the basolateral membrane (Evers *et al.*, 1996); if a similar location is

found in human small intestine then MRP1 cannot be responsible for furosemide's apparent secretion. The canalicular multispecific organic anion transporter (cMOAT, a.k.a. MRP2), which may be responsible for cisplatin resistance (Kool *et al.*, 1997), is localized in the apical membrane when expressed in Madin-Darby Canine Kidney (MDCK) cells, but has not been shown to be inhibited by indomethacin (Evers *et al.*, 1998). Definitive studies are likely to require the use of molecular biology technologies such as transgenic cell lines (Evers *et al.*, 1996; Evers *et al.*, 1998), and or "knock-out" mice (Mayer *et al.*, 1996).

Furosemide transport across both excised rat jejunum and Caco-2 cells is greater in the direction of secretion. This secretion was found to be temperature-dependent and subject to inhibition by indomethacin. These preliminary results indicate that furosemide bioavailability may be limited by an intestinal transporter.

Chapter 5

Contributions of Saturable Active Secretion, Passive Transcellular and Paracellular Diffusion to the Overall Transport of Furosemide Across Adenocarcinoma (Caco-2) Cells

5.1 Introduction

Furosemide (Lasix®) has long been known as a poorly absorbed diuretic. Extensive pharmacokinetic studies have been conducted and reviewed (Benet, 1979; Hammerlund-Udaenaes and Benet, 1989; Ponto and Schoenwald, 1990a) and the drug has seen widespread use for many years, yet the cause of its poor bioavailability remains unclear. Unsuccessful previous efforts to determine the causative factor(s) have included investigations of gastrointestinal first pass-metabolism and hydrolysis (Andreasen *et al.*, 1982; Bundgaard *et al.*, 1988; Lee and Chiou 1983), as well as dissolution limitations (Kelly *et al.*, 1974; Waller *et al.*, 1982).

Intestinal secretion was speculated to occur by many researchers (Branch, 1983; Lee and Chiou, 1983; Valentine *et al.*, 1986; Verbeeck *et al.*, 1982) to account for the extensive (approximately 50%) non-renal and non-hepatic elimination of intravenous furosemide. It was reported, however, that furosemide secretion in the small intestine does not

account for any significant elimination (Arimori and Nakano, 1988; Valentine *et al.*, 1986). The role of secretion in limiting oral bioavailability had not previously been studied. Recent findings described in Chapter 4 have demonstrated that furosemide is subject to polarized efflux in both excised rat intestine and human adenocarcinoma (Caco-2) cells (Flanagan and Benet, 1999), and in Caco-2 cells by Pade and Stavchansky (1998) two months earlier. This apparent secretion which displays temperature-dependence and sensitivity to chemical inhibition could play an important role in limiting the bioavailability of furosemide.

Here we show using Caco-2 cells, the widely used and well characterized model of human intestinal absorption (Hildago *et al.*, 1989; Hilgers *et al.*, 1990; Wilson *et al.*, 1990) with relatively few limitations (Delie and Rubas, 1997), that the observed directional difference in furosemide transport is due to saturable, active secretion. In addition, since furosemide exists almost exclusively in the ionized state with a negative charge at physiological pH (pKa= 3.9), and since the contribution of the paracellular route to overall transport through Caco-2 cells has been estimated at over 50% using a biophysical model approach (Pade and Stavchansky, 1997), the role of paracellular transport will also be investigated both qualitatively and quantitatively.

To qualitatively assess if paracellular transport is important, the free calcium in the transport media was removed using a chelating agent. EGTA has long been known to decrease transepithelial electrical resistance (TEER) and increase paracellular permeability by transiently opening tight junctions of cultured Madin Darby Canine Kidney (MDCK) cells (Martinez-Palomo *et al.*, 1980), and more recently Caco-2 cells (Artursson and Magnusson, 1990; Boulenc *et al.*, 1993). This method has been used by many researchers to assess if a compound permeates through tight junctions (Artursson and Magnusson, 1990; Boulenc *et al.*, 1993; Delie and Rubas, 1997; Gan *et al.*, 1993;

Knipp *et al.*, 1997). Our quantitative approach was analogous to that of Swaan and Tukker (1995) who compared passive permeability of Foscarnet across excised rat intestine to the paracellular marker lucifer yellow.

5.2 Materials and methods

5.2.1 Materials

The Caco-2 cell line (HTB-37), purchased from the American Type Culture Collection (ATCC, Rockville, MD), and all media were obtained from the UCSF Cell Culture Facility (San Francisco, CA). Lucifer yellow CH, lithium salt (LY) was purchased from Molecular Probes (Eugene, OR), [^{14}C]mannitol (56 Ci/mol) was purchased from New England Nuclear (Boston, MA), and all other chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

5.2.2 Transport studies

Caco-2 cells (passages 25 to 35) were cultured for studies as described in Chapter 4 (Flanagan and Benet, 1999). Briefly, studies were conducted after 21 days of growth on polycarbonate filters (0.4 μm pore size, 4.7 cm^2 area, Fisher, Pittsburgh, PA) in 6-well cluster plates. Transport at 37°C (incubator shaker) was measured in both directions after a 30 min pre-equilibration period with transport media, Minimum Essential Medium (MEM) Eagle's with 2 mM L-glutamine and Earle's BSS containing 1.5 g/L sodium bicarbonate. The permeants and inhibitors (added to both sides) were added from concentrated solutions (in DMSO, or transport media) for a final DMSO concentration in the transport media of exactly 1% and pH of approximately 7.4. For studies using EGTA, the compound was added in equal amounts by weight to both donor and receiver solutions at a final concentration of 2.5 mM. For studies using mannitol, 0.1 μCi/ml

[^{14}C]mannitol (56 Ci/mol) was added to 10 μM unlabeled mannitol in the donor solutions. Integrity of monolayers were assessed by measuring TEER using a Millipore Millicell-ERS resistance system (Millipore Corporation, Bedford, MA). Average TEER values for the Caco-2 monolayers were approximately 400 Ohms/4.7 cm^2. Sampling was done without replacement at 1 hr (100 μl), from the receiver and the entire solution was removed at 2 hr. The data in each figure or table refers to an individual experiment with its own control from a single batch (same source vial) of cells.

5.2.3 Data analysis

Net secretion was defined as the flux in the secretory B to A direction ($J_{B\ to\ A}$) divided by the flux in the absorptive A to B direction ($J_{A\ to\ B}$). Kinetic parameters were determined by fitting $J_{B\ to\ A}$ to the following equation which contains both a saturable and linear term representing the active and passive components of secretion:

$$v = [(V_{max} \times C) / (K_m + C)] + P_{pass} \times C \qquad \text{(Equation 1)}$$

where velocity (v) is analogous to flux (J), C is concentration, V_{max} is maximum flux, K_m is the apparent Michaelis constant for a carrier-mediated process, and P_{pass} is the apparent passive permeability coefficient. Sigmaplot 4.0 (SPSS, Chicago IL) was used to fit the unweighted data.

To quantitate the contribution of the paracellular route to the overall permation of furosemide we added LY and furosemide to Caco-2 cells from a single donor solution and measured the apparent permeability coefficient (P_{app}) in the two directions. Assuming that the cell behaves as a single membrane, active transport for furosemide only occurs in the secretory direction, and passive transport is independent of direction, then:

$$P_{app\ B\ to\ A} = \text{passive} + \text{active}$$

$$P_{app\ A\ to\ B} = \text{passive} - \text{active}$$

$$P_{app\ A\ to\ B} + P_{app\ B\ to\ A} = 2 \times \text{passive}$$

Thus, a measure of the passive apparent permeability can be made at any single concentration, by essentially averaging the P_{app} in the two directions:

$$(P_{app\ A\ to\ B} + P_{app\ B\ to\ A}) / 2 = \text{passive} = P_{pass} \qquad \text{(Equation 2)}$$

Statistical comparisons were performed using *t* tests (Primer of Biostatistics version 1.0, San Francisco, CA). Experimental results are presented as means ± S.D. unless otherwise noted.

5.2.4 Analytical methods

Diuretics were quantified using an LC/MS/MS system consisting of an HP1100 HPLC (Hewlett Packard, Palo Alto, CA) and PE SCIEX API300 (Perkin-Elmer Sciex Instruments, Thornhill, Ontario, Canada) mass spectrometer equipped with a TurboIonSpray source. Samples were analyzed on a C-18 column (YMC J'sphere H80 S-4, 2.0 mm i.d. x 50 mm) using a mobile phase of 0.2% formic acid/ acetonitrile (65/35) at a flow rate of 0.3 mL/min. Triamterene was analyzed under positive ionization with monitored m/z values of 254 and 237. All other diuretics were analyzed under negative ionization with monitored m/z values of 328.9 and 204.4 for furosemide, 293.6 and 213.6 for chlorothiazide, 295.8 and 268.6 for hydrochlorothiazide, 362.6 and 79.2 for bumetanide, and 221 and 82.6 for acetazolamide.

Lucifer yellow was quantified using a Cytofluor II fluorescence plate reader (Perkin-Elmer Biosystems, Foster City, CA), using an excitation wavelength of 428 nm and an emission wavelength of 530 nm. Quantitation of [^{14}C]mannitol was by liquid scintillation counting (Beckman LS1801 scintillation counter, Beckman Instruments, Inc., Palo Alto, CA.)

5.3 Results

5.3.1 Furosemide concentration dependence

To be sure that no artifacts occurred due to inherent membrane features or drug solubility, furosemide directional transport across filters-alone was measured over a concentration range from 10 to 300 μM. Membrane transport was linear with respect to time at each concentration tested (data not shown) and exhibited a linear concentration dependence (fig. 5-1). A slight directional dependence was observed at all concentrations with between 15 to 42% greater transport seen in the A to B direction, which reached significance for all but the 300 μM concentration. Similarly, permeation of the paracellular marker LY was significantly greater in the A to B direction when tested at 100 μM (10.8 ± 0.9 vs. 8.5 ± 0.5 nmol/cm^2 for the respective A to B and B to A directions). Relative rates of LY diffusion through membranes were around 40% higher than furosemide regardless of direction.

As expected, permeation across the Caco-2 cells is rate-limiting for furosemide. Overall permeation of furosemide averaged less than 10% of the filter-only controls across the entire concentration range for the B to A direction and even less for the A to B direction. Furosemide permeation through Caco-2 cells grown on filter inserts was also found to be linear with time at each concentration tested (data not shown), but unlike the membrane-only data, it was greater in the B to A direction, demonstrating significant net secretion at all concentrations (fig. 5-2). Furosemide flux as a function of concentration (from 5 to 300 μM) in the B to A direction was fit to Eq. 1 to yield a V_{max} of 437 ± 137 pmol/cm^{2}hr, K_m of 64 ± 28 μM, and P_{pass} of 3.7± 0.9 × 10^{-7} cm/s (fig. 5-2). When B to A transport was fit to Eq. 1, both saturable and non-saturable transport parameters could be obtained. Permeation in the A to B direction was best fit by a simple linear regression to yield a

P_{pass} of 3.30 ± 0.15 × 10^{7} cm/s which is slightly, but not significantly less than the P_{pass} value obtained from the B to A fit, as expected since this ignores the negative contribution of saturable secretion.

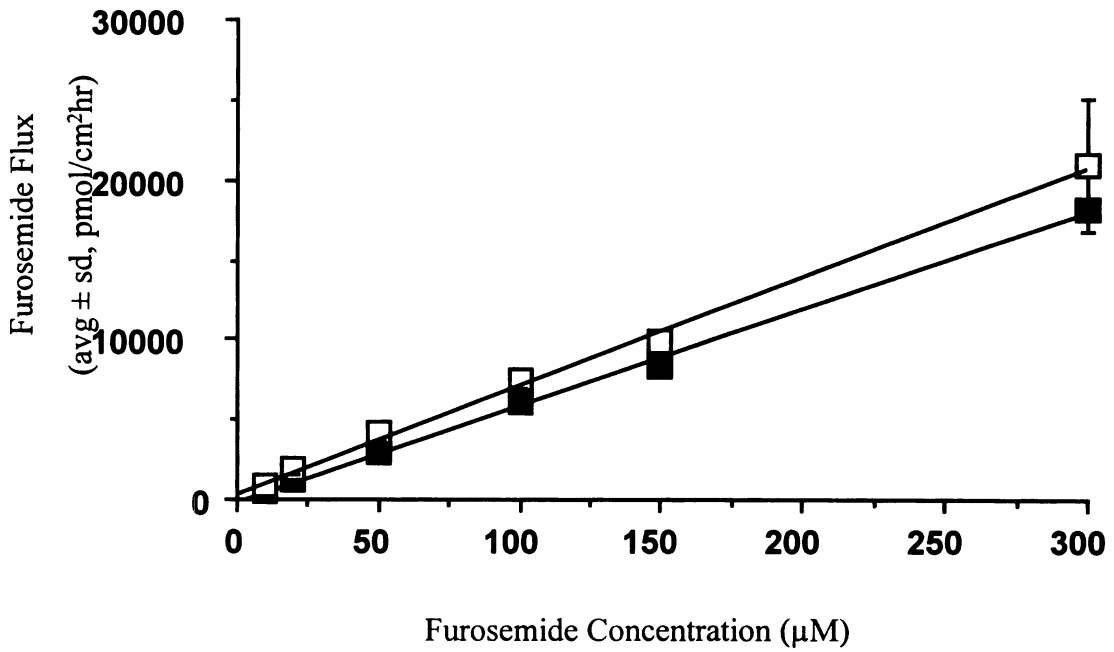


Figure 5-1: Furosemide permeation rate *vs.* concentration through 0.4 μm polycarbonate membranes. The relationship between furosemide concentration and flux through membrane filters-alone (i.e. no cells) is shown for both the B to A (■) and A to B (□) directions. Lines represent fit of linear regression (r^2= 0.998, and 0.997 for the respective directions).

5.3.2 Active transport of furosemide

In order to show that furosemide secretion is an energy dependent, and thus active process, directional transport across Caco-2 monolayers was compared in the presence and absence of the metabolic inhibitor dinitrophenol (DNP). Net secretion of furosemide was reduced by nearly 60% as a result of energy depletion, as observed by an increase in A to B transport and a decrease in B to A transport (fig. 5-3).

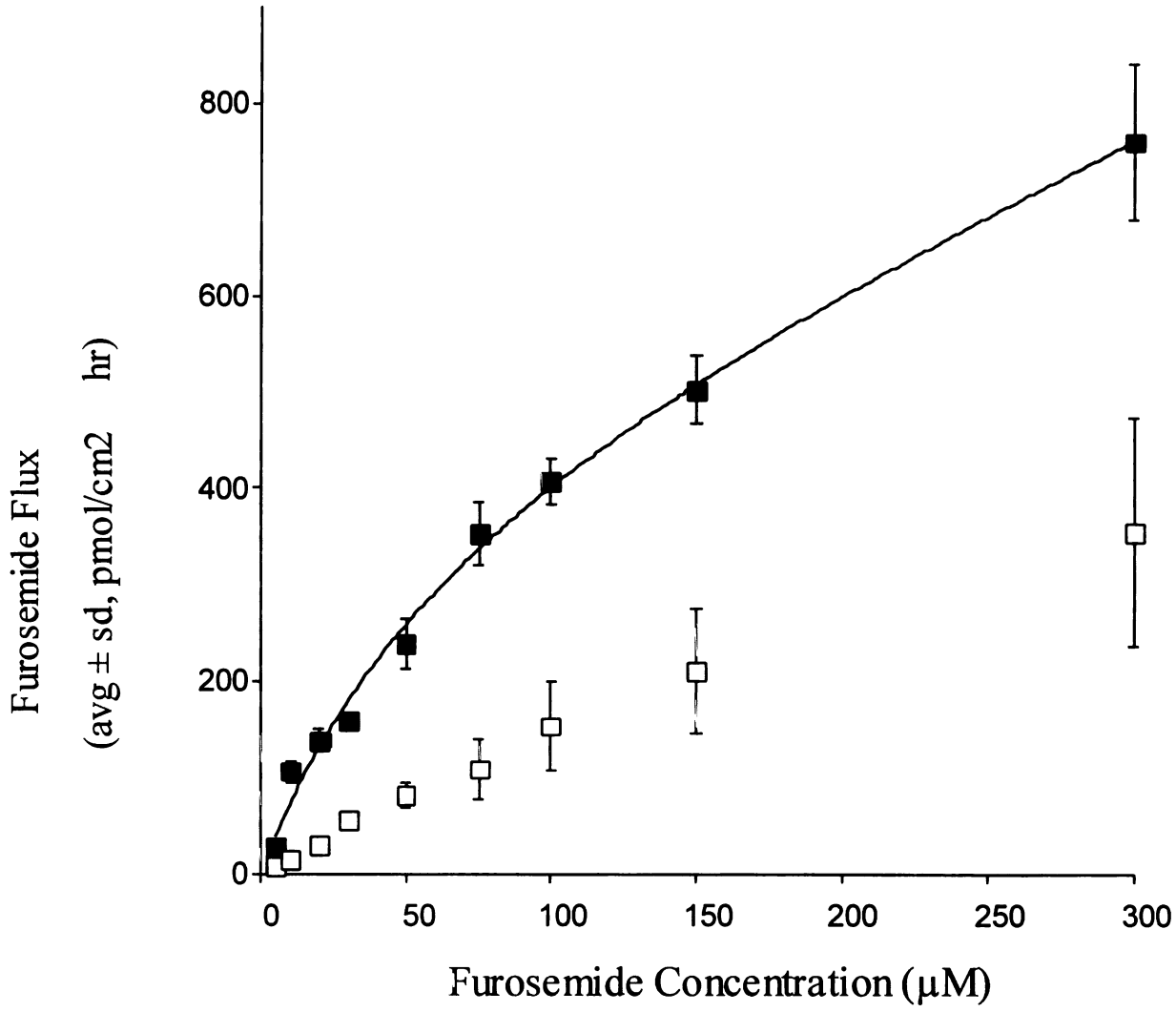


Figure 5-2: Furosemide permeation rate *vs.* concentration through Caco-2 cell monolayers. The relationship between furosemide flux and concentration through Caco-2 cells is shown for both the B to A (■) and A to B (□) directions. Solid line is fit of Eq. 1.

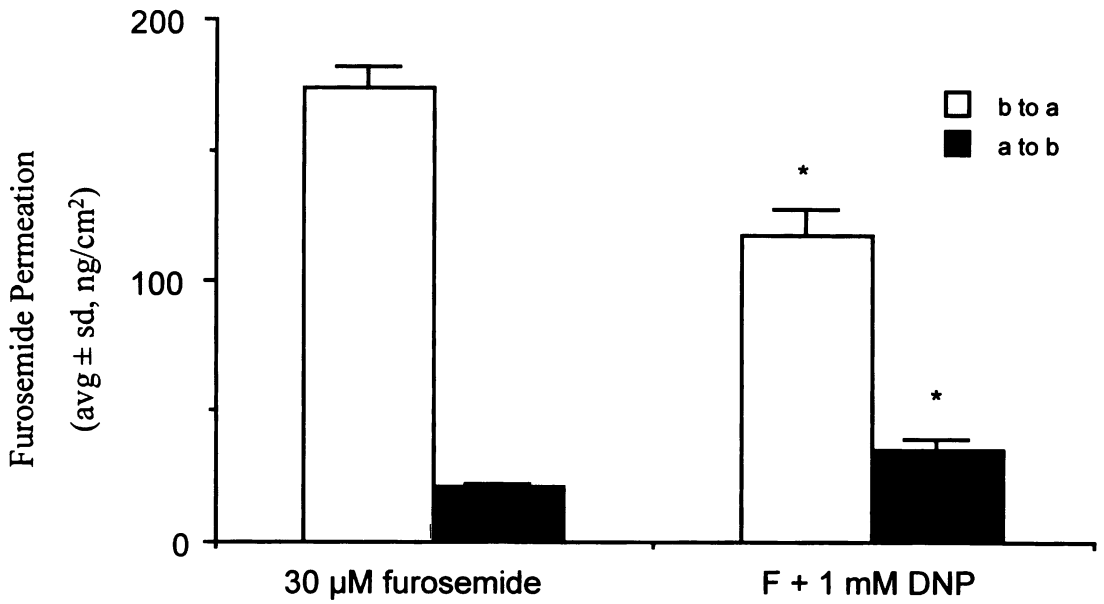


Figure 5-3: Sensitivity of furosemide transport to metabolic inhibition. The permeation of furosemide through Caco-2 epithelia in both the B to A and A to B directions was measured in the presence and absence of dinitrophenol.

* $p < 0.005$ from directional control

Another way to demonstrate active transport is to show that transport occurs against a concentration gradient, or "uphill" (Evers *et al.*, 1998). In this study, furosemide was added in equal concentrations to both sides of the Caco-2 cells in order to remove the concentration gradient that served as the driving force for passive transport. After 1 hour we find a significant difference in the concentrations on each side of the membrane, with over 15% more accumulated in the B to A direction (fig. 5-4). Naproxen was used as a control, because it is believed to be absorbed completely by passive transcellular processes (Brayden, 1997). When naproxen is added to both sides of the cells, slightly more drug accumulated in the A to B direction. There was in fact no significant difference, as expected, and the error bars are omitted to simplify the figure.

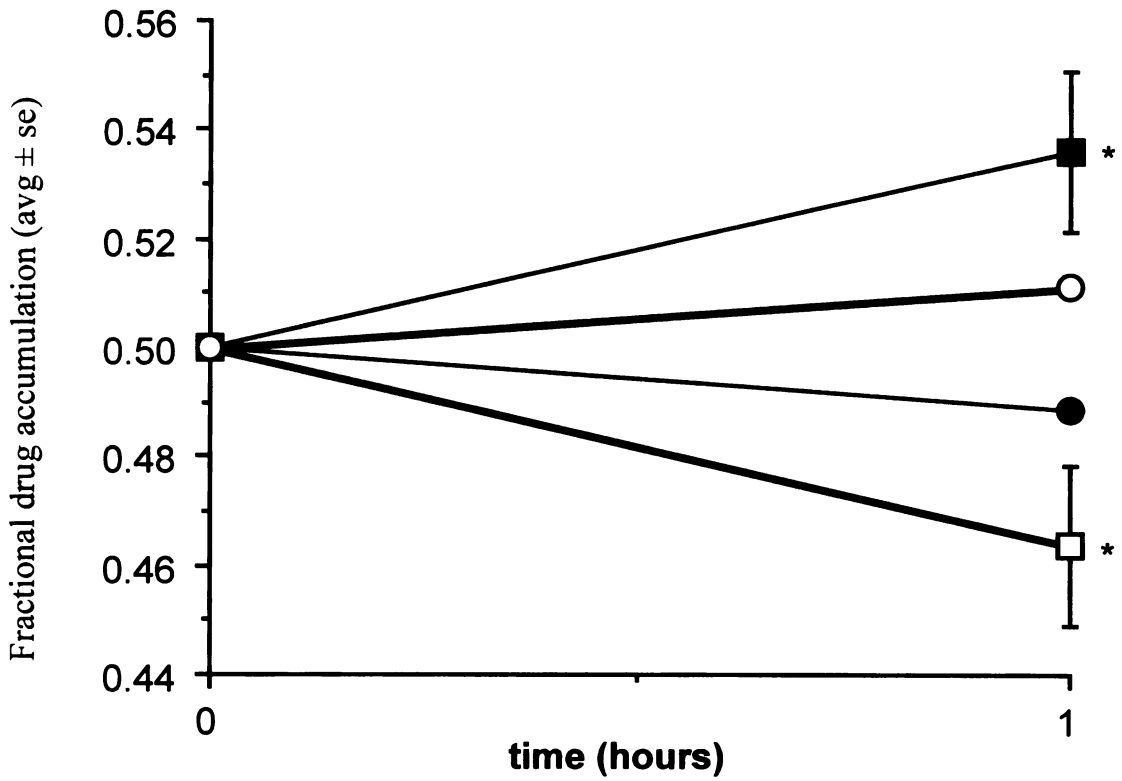


Figure 5-4: Uphill transport of furosemide in Caco-2 epithelia. The average fraction of furosemide in the apical (■) and basolateral (□) solutions was measured one hour after loading an equal concentration (1 μM) to both sides of Caco-2 monolayers. This was repeated for naproxen (apical ●, and basolateral ○) also at 1 μM, error bars not shown. * $p < 0.05$ for the two directions

5.3.3 Chemical inhibition of furosemide transport

Indomethacin and probenecid, known inhibitors of organic anion transport, have previously been shown to inhibit furosemide secretion by Caco-2 cells (Flanagan and Benet 1999). A few other anionic compounds were tested for their ability to effect directional transport of furosemide. Sulfinpyrazone was found to markedly reduce secretion in the B to A direction to just 22% of the control value and also caused a

smaller (70% of control) decrease in the A to B direction (table 5-1). In this study sodium salicylate had no significant effect on B to A transport, but was able to increase A to B transport significantly, whereas uric acid did not change furosemide permeation in either direction.

Table 5.1: Effect of anionic inhibitors on the secretion of furosemide by Caco-2 cells. Various inhibitors were added to both sides of the membrane at 1 mM concentration to study their effect on directional permeation of 30 μM furosemide through Caco-2 cells. Testing was in triplicate for the 2 hour study.

Inhibitor	Cumulative permeation *ng cm*$^{-2}$		Net secretion
	B to A	A to B	
control	105.2 ± 5.2	18.9 ± 0.8	5.6
uric acid	117.6 ± 9.0	17.7 ± 4.0	6.7
sodium salicylate	115.8 ± 7.8	26.6 ± 2.6**	4.4
sulfinpyrazone	23.1 ± 5.8***	13.2 ± 2.8*	1.8

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$

5.3.4 Paracellular transport of furosemide

To assess the contribution of paracellular transport to furosemide permeation, 2.5 mM of the calcium chelator EGTA was added to both sides of the Caco-2 monolayers. This concentration of EGTA is sufficient to open cellular tight junctions of Caco-2 cells (Artursson and Magnusson, 1990; Boulenc *et al.*, 1993). Transport was increased in the

presence of EGTA indicating that the paracellular route may contribute to the absorption of furosemide (fig. 5-5). This increase was greatest for the A to B direction (14-fold vs. only 2-fold increase in the B to A direction) reducing the overall net secretion. It is important to note however, that even when the tight junctions are opened, there is still a significant net secretion of furosemide suggesting that the energy dependent secretion is the dominant pathway.

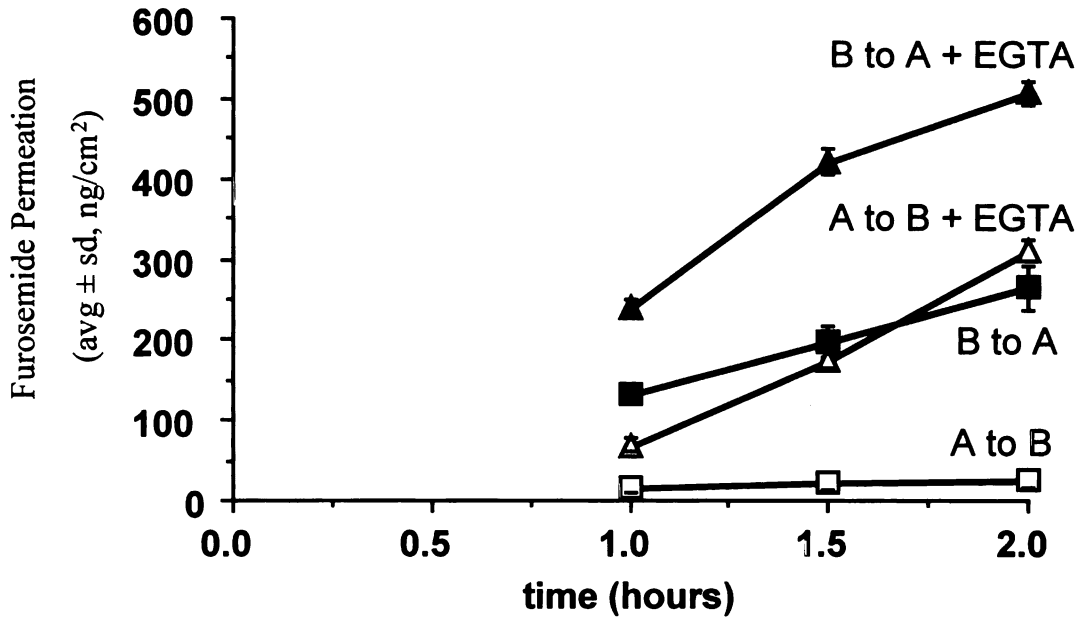


Figure 5-5: Effect of calcium chelation on permeation of furosemide through Caco-2 epithelia. The permeation of furosemide (30 μM) in the B to A (■, ▲) and A to B (□, Δ) directions was determined in the presence (▲, Δ) and absence (■,□) of 2.5 mM EGTA.

In a separate study, the permeation of furosemide and LY, a marker for paracellular transport, was compared from the same donor solution (table 5-2). Controls within the study showed that LY and furosemide had no effect on each others permeation through Caco-2 cells (data not shown). Approximately 30% more LY permeated in the B to A direction, but this wasn't significant due to high variation in the A to B direction. This

variation in LY did not correlate with furosemide variation, which is in accordance with our general observation that leaks (validated by decreases in TEER values and increases in permeation of other molecules) are usually associated with several fold increases in LY values. Furosemide permeation did show a significant net secretion of over 6-fold.

Table 5-2: Comparison of LY and furosemide directional permeability through Caco-2 cells. Apparent permeability coefficients are shown for diffusion of lucifer yellow (100 μM) and furosemide (30 μM), loaded together, through Caco-2 monolayers. Testing was in triplicate for the 2 hour study.

Permeant	$P_{app} \times 10^{-7}$ *cm/s* (CV)	
	B to A	A to B
Lucifer Yellow	3.12 ± 0.36 (12%)	2.39 ± 2.18 (91%)
Furosemide	14.97 ± 7.01 (47%)	2.29 ± 0.89* (39%)

* $p < 0.05$ for directional difference

The effect of EGTA was evaluated on furosemide and LY concomitant transport, as well as mannitol in separate plates of the same source cells (fig. 5-6). In all cases, EGTA significantly increased transport irrespective of direction. Similar magnitudes of EGTA enhancement for the A to B and B to A respective directions were observed for both LY (8.7- to 11.3-fold) and mannitol (3.6- to 4.2-fold), whereas the effect of EGTA again showed directional dependence for furosemide (9.4- to 2.5-fold). Another key difference between the effect of EGTA on furosemide vs. the paracellular marker compounds is in terms of net secretion (fig. 5-7). No directional difference was seen for LY or mannitol which permeated in the two directions at essentially a 1:1 ratio, as expected, and net

secretion of both compounds showed only a slight increase in the presence of EGTA. EGTA had a much greater effect on furosemide secretion (and in the opposite direction) reducing it enough in this study to eliminate any significant directional difference.

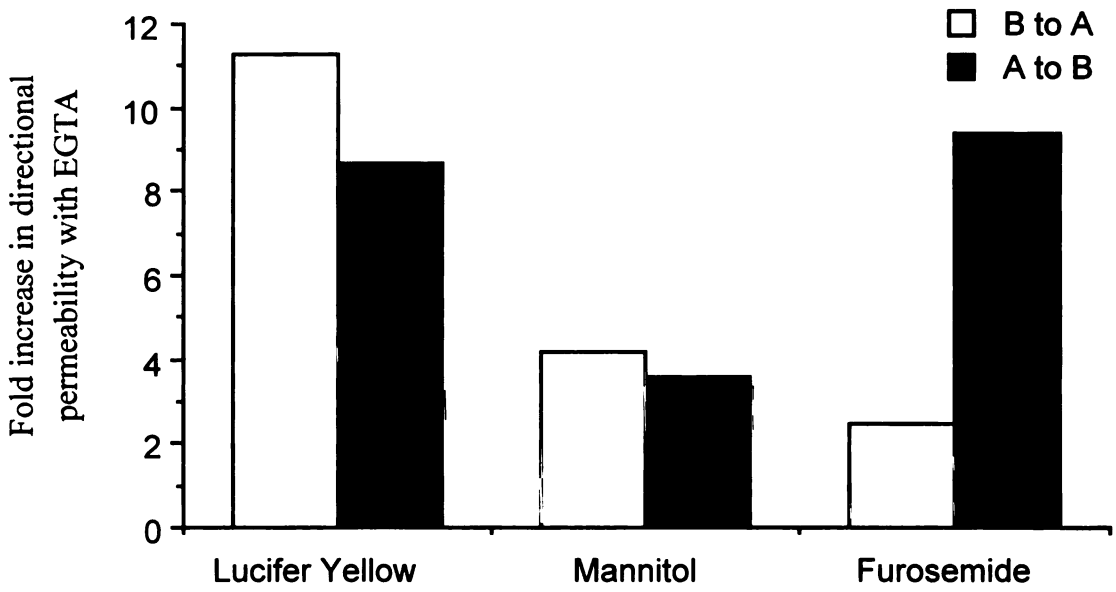


Figure 5-6: Effect of calcium chelation on directional transport through Caco-2 epithelia. The addition of 2.5 mM EGTA caused a significant increase in the Caco-2 permeation of LY (100 μM), mannitol (10 μM) and furosemide (30 μM). The magnitude of this increase is shown for both the B to A and A to B directions for these three compounds.

5.3.5 Secretion of other diuretics

In order to determine if intestinal secretion was a general phenomenon for other diuretics, five additional diuretics were tested in the Caco-2 model (table 5-3). Four of the five diuretics tested, which included thiazide (chlorothiazide and hydrochlorothiazide), loop (bumetanide), potassium-sparing (triamterene) and carbonic anhydrase inhibitor (acetazolamide) classes, showed net secretion with significance reached in three cases. Only the carbonic anhydrase inhibitor, acetazolamide, whose bioavailability is nearly complete (Jackson, 1996) was not secreted in this intestinal model.

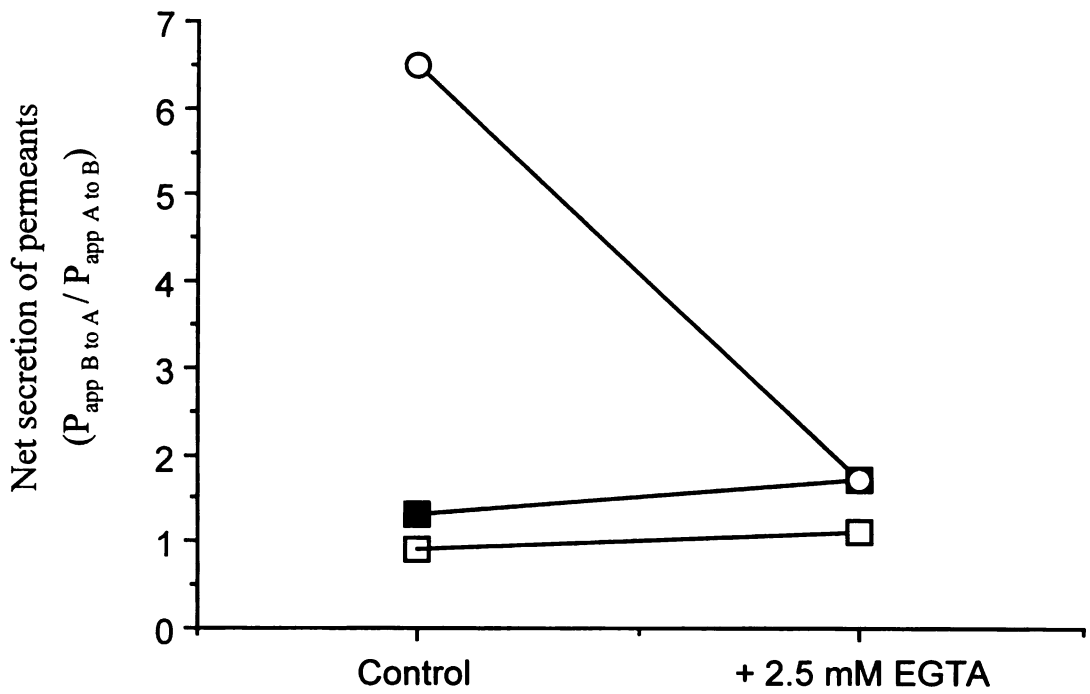


Figure 5-7: Effect of calcium chelation on net secretion by Caco-2 epithelia. The effect of 2.5 mM EGTA on net secretion by Caco-2 cells is shown for LY (■), mannitol (□) and furosemide (○).

Table 5-3: Permeation of diuretics through Caco-2 epithelia. Directional permeation through Caco-2 cells of various diuretics (100 μM) were measured. Testing was in triplicate for the 2 hour study.

Diuretic	$P_{app} \times 10^{-7}$ *cm/s*		Net secretion
	B to A	A to B	
acetazolamide	1.26 ± 0.04	1.32 ± 0.14	1.0
bumetanide	0.21 ± 0.01	0.09 ± 0.01	2.4***
chlorothiazide	0.44 ± 0.03	0.28 ± 0.01	1.6***
hydrochlorothiazide	1.76 ± 0.55	1.30 ± 0.40	1.4
triamterene	9.58 ± 0.11	3.50 ± 0.12	2.7***

*** $p < 0.001$

5.4 Discussion

In Chapter 4, furosemide secretion from Caco-2 cells was shown to be temperature-dependent and sensitive to inhibition by a variety of compounds including indomethacin (Flanagan and Benet, 1999). This led to the conclusion that a carrier-mediated transport process occurs for furosemide which we hypothesize to contribute to its poor bioavailability. Before initiating a clinical study (described in Chapter 7) to test that hypothesis, we wanted to further characterize the transport of furosemide across Caco-2 cells in terms of its concentration and energy-dependence, and to investigate the potential contribution of the paracellular route to total transport across these cells. The results of these *in vitro* experiments, summarized below, served as the justification for the *in vivo* human study.

Analysis of furosemide (fig. 5-1) and LY directional transport across membrane filters alone, showed a linear dependence on concentration for both directions with slightly greater permeation in the A to B direction. This directionality could be due to hydrostatic forces intrinsic to the system design. The permeation is greater from the top (apical) to the bottom (basolateral) chamber and correlates with accumulation of fluid over time into the bottom compartment as well. This result established that any artifacts due to the filters alone, physiochemical properties of the drug, or membrane properties would tend to exaggerate A to B and reduce B to A transport.

Furosemide permeation across Caco-2 cells showed a significant secretory preference and a saturable dependence on concentration in the B to A direction which could be fit to Eq. 1 (fig. 5-2). Fitting data to both linear and saturable terms, which is common when analyzing uptake studies (Tamai *et al.*, 1997), is not preferred for transport studies where the insignificant passive permeation is sometimes ignored (Zhang and Benet, 1998) or factored out by fitting a difference between the two directions (Hunter *et al.*, 1993;

Karlsson *et al.*, 1993). Apparently linear permeation in the direction opposite of transport (A to B) such as that observed in this study, has been reported by some researchers (Swann and Tukker, 1995), while others have seen saturable secretion in both directions (Karlsson *et al.*, 1993).

It has been shown that DNP can block energy-dependent transport processes in Caco-2 (Dantzig *et al.*, 1994) and LLC-PK1 (Ito *et al.*, 1993) cells. Our results show that DNP inhibits secretion of furosemide significantly causing both an increase in A to B and decrease in B to A transport (fig. 5-3). Active transport of furosemide was also shown by demonstrating uphill transport. By adding equal concentrations of drug to both sides of the membrane, the concentration gradient that provides the driving force for passive transport is removed. Any transport under those conditions can only be caused by active processes. The transcellular marker naproxen had a slight insignificant accumulation in the A to B direction (fig. 5-4), which supports our findings using membranes alone (without cells) that permeants tend to pass from the top (apical) chamber down to the bottom (basolateral) chamber. Actual transport data for naproxen across Caco-2 cells showed no directional difference (Pade and Stavchansky, 1998). Furosemide did show accumulation in the apical chamber indicating active transport in the B to A direction (fig. 5-4).

This active secretion could be the result of either a primary active efflux pump such as the Multidrug Resistance-associated Protein (MRP), or a secondary active transporter such as a member of the organic anion transporter (OAT) family. In Chapter 4, chemical inhibitors were used to try to identify a specific transport system (Flanagan and Benet, 1999). This approach failed to implicate a single transporter primarily because of the lack of specificity of inhibitors. Compounds that inhibited both non-specific organic anion transport and multidrug resistance protein (MRP-1), or both P-glycoprotein (P-gp)

and MRP-1 were found to cause a decrease in furosemide secretion. This overlap would have lead to the belief that MRP-1 was responsible for furosemide's secretion, were it not for the fact that MRP-1 is expressed on the basolateral surface (in overexpressing LLC-PK1 cells, Evers *et al.*, 1996) and would be expected to result in net absorption of furosemide. The canalicular Multispecific Organic Anion Transporter (cMOAT, a.k.a. MRP-2) points in the right direction- is expressed on the basolateral membrane, but is not thought to be sensitive to indomethacin inhibition (Evers *et al.*, 1998). Preliminary results using these and other transfected cell lines confirm that furosemide is not transported by MRP-1, cMOAT (MRP-2), or P-gp (see Chapter 6).

Since both Caco-2 and *in vivo* renal secretion of furosemide is decreased with coadministration of the uricosuric agent probenecid, the effects of another anti-gout compound (and known inhibitor of MRP-1) sulfinpyrazone was tested. Uric acid, whose active reabsorption is blocked by probenecid and sulfinpyrazone, and sodium salicylate, which also interacts with both drugs and uric acid at high doses, were also tested (table 5-1). Sodium salicylate increased A to B permeation presumably by blocking secretion, and also caused a slight, insignificant increase in the B to A direction. Sulfinpyrazone decreased B to A transport markedly, but also caused a decrease in A to B permeation. General decreased permeability caused by sulfinpyrazone, or general increases due to sodium salicylate unrelated to interactions with transport proteins have not been reported. Furthermore, there was no change in the permeation of the paracellular permeation marker LY with any inhibitor treatment. One explanation for these effects could be the presence of multiple transporters with varying degrees of susceptibility to inhibition that combine to result in a net secretion, but may not all function as secretory pumps.

There is some concern that Caco-2 cells may not be the best model for studying paracellular transport. Permeability of hydrophilic compounds across these cells was

found to more closely approximate their tissue of origin (colon) than small intestinal cells (from rat, Collette *et al.*, 1997; Duizer *et al.*, 1997), due to less, not tighter, junctions per unit area (Tanaka *et al.*, 1995). Still, it has been reported that Caco-2 cells are useful for predicting human absorption regardless of pathway (Yee, 1997). Furthermore, this cell line has been used extensively to study paracellular transport both qualitatively (Boulenc *et al.*, 1993; Gan *et al.*, 1993; Delie and Rubas, 1997; Knipp *et al.*, 1997) and quantitatively (Pade and Stavchansky, 1997). We decided to study paracellular transport in the same cell line (Caco-2) used to accomplish our other objectives for lack of a better established alternative.

Qualitatively, paracellular permeation is important for furosemide given the increases in permeation under conditions that open tight junctions (fig. 5-5). Even though EGTA caused an increase in the permeability of furosemide and the paracellular markers LY and mannitol, only furosemide had a large directional difference in the magnitude of the increase whereas the changes in the paracellular marker where roughly equal in the two directions (fig. 5-6). The result of this directional susceptibility of furosemide to EGTA is a reduction in net secretion while the paracellular markers remain mostly unchanged (fig. 5-7). It appears that furosemide behaves differently than a pure paracellular compound.

When the passive component of furosemide transport is calculated by Eq. 2 for the data in table 5-2, a P_{pass} of 8.6×10^{7} cm/s is obtained. This is greater than the value we derived earlier using a wide range of concentrations (3.7×10^{7} cm/s), but is of similar order of magnitude and within the range of values we typically observe. More importantly for the purposes of comparison, it is the value that we obtained in the same cells in which LY permeation was determined. If we assume that LY permeates exclusively through the tight junctions, at a rate similar to that of furosemide (since permeation of both LY and

furosemide across filters alone is similar) then LY permeation represents the maximum rate through the paracellular path (Swaan and Tukker, 1995). Since, as expected, no directional difference was seen for LY, the P_{app} is the same as the P_{pass}. Comparison of the calculated P_{pass} for furosemide to the permeability of LY across the same cells allows us to infer that between 27 to 36% (32%) permeates through the tight junctions depending on whether you compare to the A to B, B to A or (mean) LY P_{app}, respectively. By this analysis, approximately one-third of the passive furosemide permeation occurs through the paracellular route, which is fairly consistent with the theoretical value of approximately one-half obtained by Pade and Stavchansky (1997), and is still consistent with overall secretory transport.

Given that in the case of furosemide, renal secretion was predictive of intestinal secretion, we decided to determine if intestinal secretion occurs for a series of diuretics that are transported in the kidneys. The potassium-sparing diuretic, amiloride, was shown in Chapter 3 to have net secretion of 2- and 5-fold across Caco-2 monolayers and excised rat jejunum, respectively (Flanagan and Benet, 1998). Greater net secretion from Caco-2 cells has been observed for chlorothiazide (over 8-fold greater B to A, Pade and Stavchansky, 1998). Triamterene, bumetanide (another loop diuretic shown in Chapter 4 to inhibit furosemide transport, Flanagan and Benet, 1999), and two thiazide diuretics were tested together with acetazolamide. The majority of these diuretics showed small net secretion (1.4- to 2.7-fold), that reached significance for all but one compound (hydrochlorothiazide), while acetazolamide exhibited no secretion (table 5-3). Although renally eliminated, acetozolamide is the only diuretic tested that does not interact with a membrane bound transporter or channel for its diuretic effect. Bioavailability of these diuretics varied from 15-25% for chlorothiazide, 59-89% for bumetanide and acetazolamide's absorption is nearly complete (Jackson, 1996). Additional studies need to be conducted to determine if secretion of these compounds plays a role in their

bioavailability. We conclude that furosemide secretion from Caco-2 cells is the result of saturable active transport and linear passive diffusion that has a significant paracellular component.

Chapter 6

Furosemide Secretion from Cell Lines Overexpressing Multidrug Resistance Protein (P-glycoprotein) and Multidrug Resistance-associated Proteins (MRP-1 and MRP-2)

6.1 Introduction

Results from Chapters 4 and 5 have demonstrated that the secretion of furosemide is a carrier-mediated and energy dependent process. Furthermore, this secretion is subject to chemical inhibition and could be the result of a membrane-bound transport protein. Determination of a specific transporter was not possible using chemical inhibitors because of the substrate promiscuity of these transporters. Recent recombinant techniques have allowed the use of polarized cell lines overexpressing human cDNA to study the effect of specific transporters, such as P-gp. Three transporters known to pump a wide range of compounds out of cells, P-gp in MDCK (strain I), MRP2 (cMOAT) in MDCK (strain II) and MRP1 in LLC-PK1 cells, are studied in this chapter for their ability to transport furosemide. In addition, studies on the temperature and concentration dependence of furosemide transport in the MDCK cell line, as well as chemical inhibition studies in all cell lines, are presented.

6.2 Materials and methods

6.2.1 Materials

All cell culture media was obtained from the UCSF Cell Culture Facility (San Francisco, CA). P-glycoprotein transfected and wild-type Madin-Darby Canine Kidney, strain I (MDR1-MDCK and MDCK, respectively) were generously provided by Dr. Ira Pastan (NIH, Bethesda, MD). MRP1 (LLC-MRP1) and MRP2 (MDCKII-cMOAT), as well as the respective wild-types (LLC-PK1 and MDCK strain II) were generously provided by Dr. Piet Borst at the The Netherlands Cancer Institute (Amsterdam, The Netherlands). Cyclosporine was a gift from Sandoz Pharmaceuticals (Basel, Switzerland). Lucifer yellow CH, lithium salt (LY) was purchased from Molecular Probes (Eugene, OR). Radiolabeled compounds were purchased from New England Nuclear (Boston, MA), [^{14}C]mannitol (56 Ci/mol), and Amersham Pharmacia Biotech (Buckinghamshire, England), [^{3}H]vinblastine (15.5 Ci/mmol) and [^{3}H]cyclosporine (8.3 Ci/mmol). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

6.2.2 Preparation of cell culture monolayers and transport studies

All cells were cultured at 37 °C and humidified, 5% CO_2-atmosphere in medium containing 10% fetal bovine serum. Transfected and wild-type MDCK cells (strains I and II), were grown in Dubelco's modified Eagle's medium (DMEM). The P-gp transfected (MDR1-MDCK) cells also contained 80 ng/ml colchicine to maintain transfection. The MRP2 transfected (cMOAT-MDCK) and wild-type MDCKII cells as well as the MRP1 transfected (LLC-MRP1) and wild-type LLC-PK1 cells all contained antimicrobial agents (100 µg/ml streptomycin and 100 units/ml penicillin G), as advised by Dr. Raymond Evers (personal communication). The wild-type and transfected LLC-PK1 cells were grown in Medium 199 with Earle's salts, L-glutamine and 2.2 mg/L sodium bicarbonate. Cells grown to confluence in culture flasks were harvested with

0.05% Trypsin EDTA and seeded onto polycarbonate filters (0.4 μm pore size, Fisher, Pittsburgh, PA) in 6-well cluster plates at an approximate density of 10^6 cells/insert. Studies were conducted at 4 to 5 or 4 to 6 days post seeding for the four MDCK or two LLC-PK1 cell lines, respectively. Media was changed once every 2 days for all of the cell lines and always included feedings 18 to 24 hr both post-trypsinization and pre-testing.

Studies were conducted after equilibrating the cells at 37 °C for 30 min in serum free growth media without any additional supplements (colchicine or antibiotics). Volume of the chambers was 1.5 ml on the apical side and 2.5 ml on the basolateral side. Both the basolateral-to-apical and apical-to-basolateral directions were tested over a surface area of 4.71 cm^2. Furosemide and inhibitors (added to both sides) were added from concentrated DMSO solutions for a final solvent concentration in the transport media of exactly 1% and pH of approximately 7.4. Studies were conducted at 37°C (incubator shaker), unless otherwise noted. Integrity of monolayers were assessed by measuring transepithelial electrical resistance (TEER) using a Millipore Millicell-ERS resistance system (Millipore Corporation, Bedford, MA).

Lucifer yellow (LY) and mannitol were used as markers of paracellular permeability in some cell lines as noted. Studies using LY (150μM) in MDCK cells were conducted in Hank's balanced salt solution because the DMEM was found to interfere with the LY quantitation. Permeation of mannitol and cyclosporine were measured using donor solutions containing both radiolabeled and unlabeled compound for final concentrations of 10 μM (0.1 μCi/ml) and 5 μM (1 μCi/ml), respectively. Donor solution of [^{3}H]vinblastine sulphate was prepared exclusively in the radiolabeled form to final concentrations of 35 to 50 nM (0.54 to 0.78 μCi/ml).

Sampling was done without replacement at various time points during the experiments from the receiver and the entire solution was removed at 2 hr. The maximum volume removed during the course of an experiment was 0.15 ml, which corresponds to 10% of the total volume of the apical and 6% of the basolateral starting volumes. The data in each figure or table refers to an individual experiment with its own control from a single batch (same source vial) of cells.

6.2.3 Analytical methods

Furosemide was quantified using an LC/MS/MS system consisting of an HP1100 HPLC (Hewlett Packard, Palo Alto, CA) and PE SCIEX API300 (Perkin-Elmer Sciex Instruments, Thornhill, Ontario, Canada) mass spectrometer equipped with a TurboIonSpray source. Samples were analyzed on a C-18 column (YMC J'sphere H80 S-4, 2.0 mm i.d. x 50 mm) using a mobile phase of 0.2% formic acid/ acetonitrile (65/35) at a flow rate of 0.3 mL/min. Positive ionization mode was used with monitored m/z values of 328.9 and 204.4.

Lucifer yellow was quantified using a Cytofluor II fluorescence plate reader (Perkin-Elmer Biosystems, Foster City, CA), using an excitation wavelength of 428 nm and an emission wavelength of 530 nm. Quantitation of [^{14}C]mannitol, [^{3}H]vinblastine and [^{3}H]cyclosporine was by liquid scintillation counting (Beckman LS1801 scintillation counter, Beckman Instruments, Inc., Palo Alto, CA.)

6.3 Results

6.3.1 Paracellular permeability of the transfected cell lines

Average TEER values were determined during the experiments for all of the cell cultures tested. Typical values (shown in table 6-1) varied between cell lines and between wild-type and transfected cells, where the largest differences were seen. Cells transfected with P-gp or MRP1 were nearly three-times more resistant (tighter junctions) than the wild-types. In contrast, no difference was seen for the cMOAT transfected cells whose TEER values were close to the TEER values measured across filters alone (120-150 ohms/4.7 cm^2) and not different from their low resistance parental strain.

Table 6-1: Transepithelial resistances of various cell lines. Cells were seeded at similar seeding densities for all cell lines. The TEER values were taken immediately prior to transport experiments, 4 to 6 days after seeding the cells.

Cell line	TEER mean (range) in Ohms/4.7 cm^2
MDCK (strain I)	270 (220-305)
P-gp transfected	760 (620-905)
MDCK (strain II)	160 (150-170)
MRP2 transfected	160 (150-175)
LLC-PK1	195 (175-210)
MRP1 transfected	530 (475-585)

Permeability of the paracellular markers mannitol and LY was also tested in some of the cell lines. Mannitol transport across LLC-PK1 and LLC-MRP1 cells was found to be both independent of direction and extremely low permeating. In both cases less than 0.01% was recovered in the receiver from 50 μM donor solutions (data not shown). Absolute permeability to mannitol was about twice as high in the wild-type cells which is consistent with the observed TEER differences.

The permeation of LY was tested in both directions across cells transfected with MRP1, MRP2 and their respective wild-types as shown in table 6-2. MDCKII cells were more permeable than LLC-PK1 cells as reflected by the differences in TEER values and no directional differences were observed. No correlation between TEER differences and LY permeabilities were observed when comparing the parental to transfected cell lines. Peremation of LY across LLC-PK1 and LLC-MRP1 cells were the same, and across MDCKII and MDCKII-cMOAT were quite different despite opposite TEER relationships.

Table 6-2: Apparent permeability coefficients of LY across selected cell lines.

	Lucifer Yellow $P_{app} \times 10^{-7}$ cm/s	
Cell line	B to A	A to B
MDCK (strain II)	12.2 ± 3.9	7.7 ± 1.7
MRP2 transfected	57.2 ± 9.4	51.6 ± 9.3
LLC-PK1	1.0 ± 0.3	2.2 ± 2.0
MRP1 transfected	1.3 ± 0.4	1.8 ± 1.0

6.3.2 Secretion of vinblastine by wild-type vs. transfected cell lines.

Vinblastine was found to be secreted by both MDCKII wild-type and MDCKII-cMOAT cells (fig. 6-1). Permeability of MRP2 transfected cells to vinblastine was greater than in the wild-type cells. The magnitude of this increase varied slightly between the A to B (5-fold increase) and B to A (3-fold increase) directions, resulting in an unexpected reduction in overall secretion in the overexpressing cell line (8- vs. 14-fold). This secretion was found to be greatly inhibited by cyclosporine and slightly increased by sulfinpyrazone in the MDCKII cells (fig.6-2). In contrast, sulfinpyrazone treatment resulted in an inhibition of vinblastine secretion by MRP2 transfected cells, whereas

cyclosporine caused a decrease in secretion (shown only by an increase in A to B permeation) in this cell line as well (fig. 6-3).

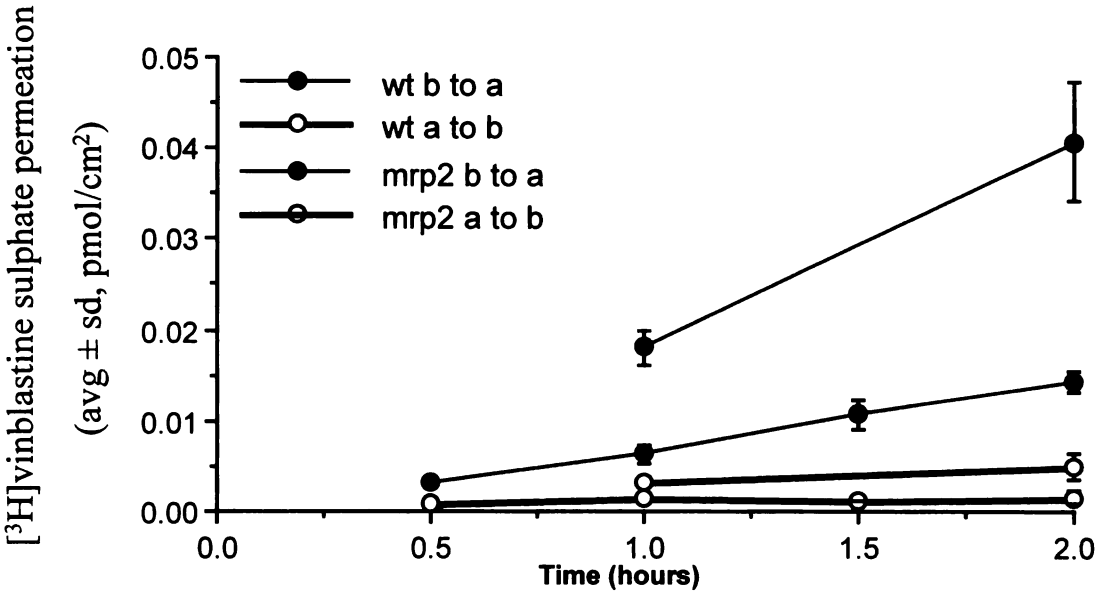


Figure 6-1: Permeation of vinblastine (35 nM) across MDCKII wild-type and MRP2 transfected cells.

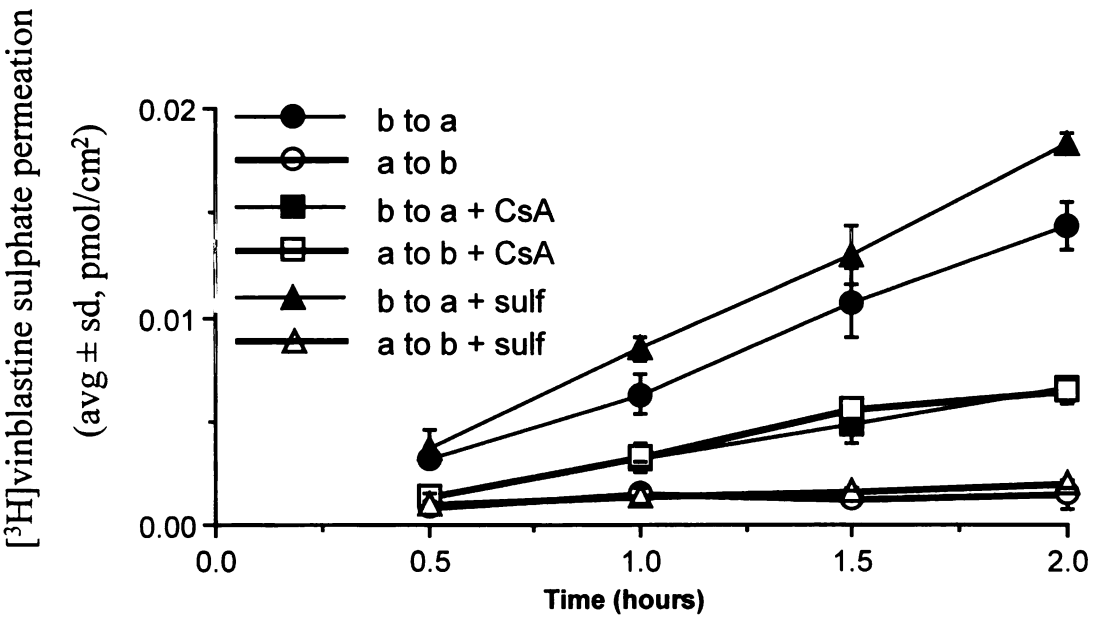


Figure 6-2: Effect of inhibitors, cyclosporine (CsA, 20 μM) and sulfinpyrazone (sulf, 1mM), on vinblastine permeation (35 nM) across MDCKII cells.

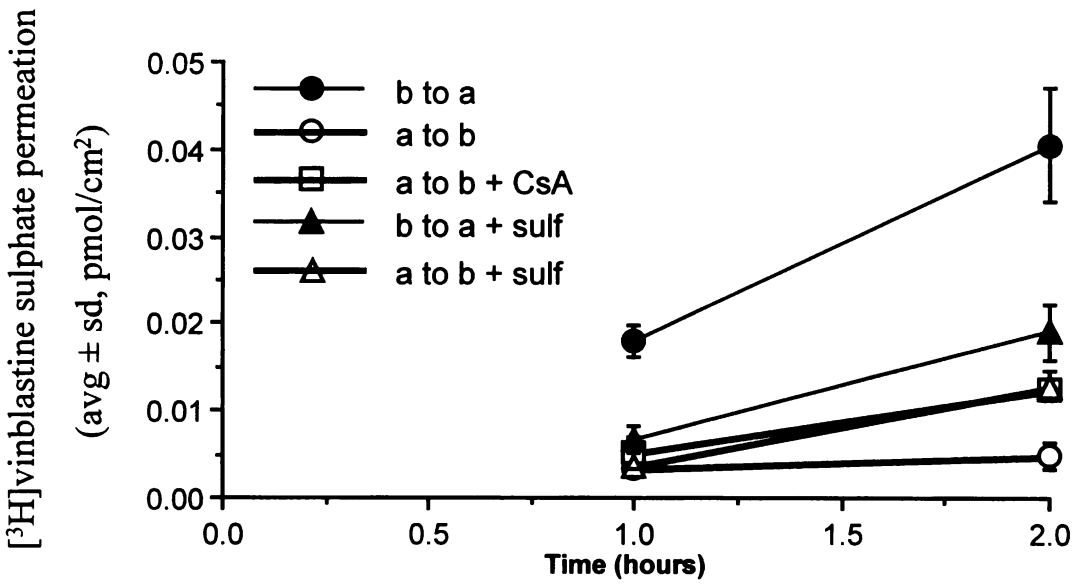


Figure 6-3: Effect of inhibitors, cyclosporine (CsA, 20 μM) and sulfinpyrazone (sulf, 1mM), on vinblastine permeation (35 nM) across MDCKII-MRP2 cells. The B to A results for cyclosporine treatment had extremely large standard deviations and were omitted from this figure.

Permeation of vinblastine (50nM) by MRP-1 transfected cells was slightly greater in the B to A direction and significantly lower in the A to B direction than for the wild-type LLC-PK1 cells (fig.6-4). Overall net secretion was observed in both cell lines, but was twice as high in MRP-1 transfected cells (6- vs. 3-fold). A decrease in net secretion was observed in wild-type LLC-PK1 cells with the addition of cyclosporine (not shown). A similar decrease with cyclosporine addition was found in cells transfected with MRP (from 4- down to 1.4-fold), whereas sulfinpyrazone caused a small increase (up to 4.6-fold) in the vinblastine secretion by these cells only (fig. 6-5).

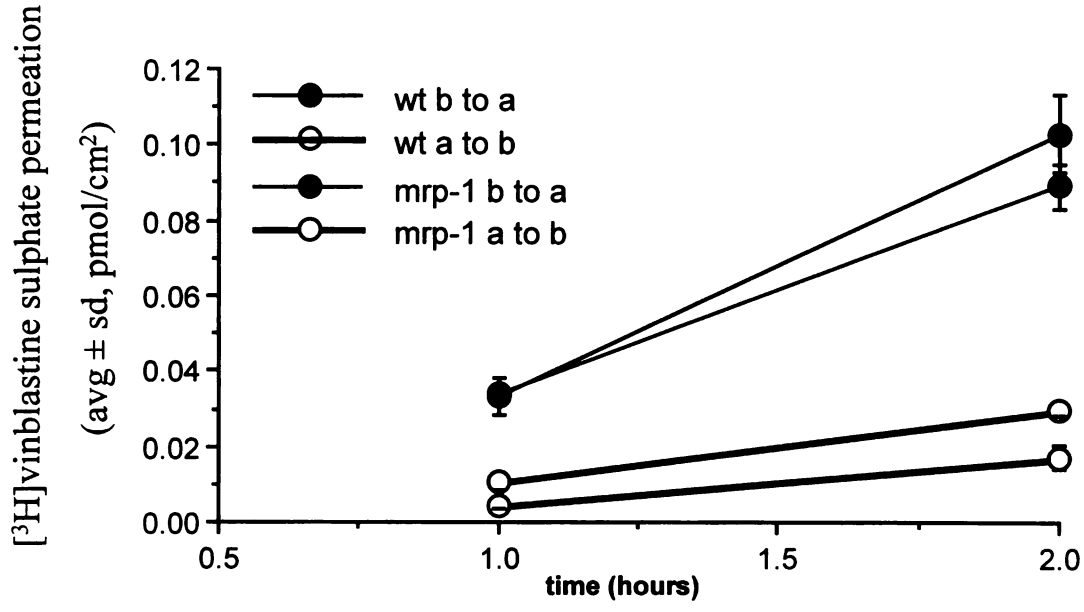


Figure 6-4: Permeation of vinblastine (50 nM) across LLC-PK1 wild-type and MRP1 transfected cells.

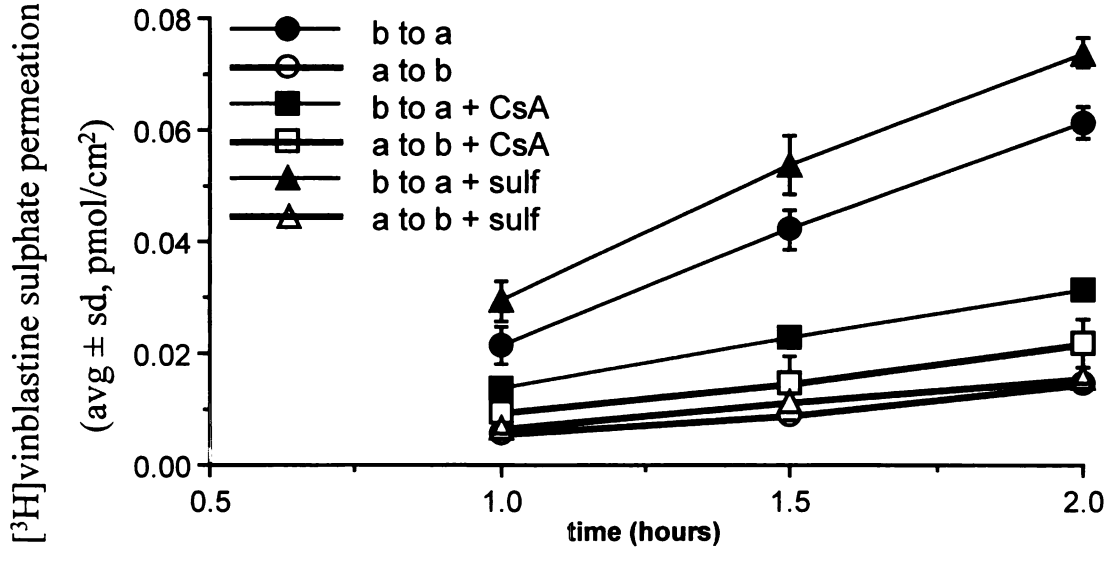


Figure 6-5: Effect of inhibitors, cyclosporine (CsA, 20 μM) and sulfinpyrazone (sulf, 1mM), on vinblastine permeation (35 nM) across LLC-MRP1 cells.

6.3.3 Furosemide secretion by wild-type vs. transfected cell lines.

Furosemide was secreted by all cell types tested. This secretion decreased slightly in cells overexpressing cMOAT vs wild type MDCKII cells (fig. 6-6), and was the same in both the LLC-PK1 and overexpresing MRP1 cell lines (fig 6-7). Similarly, secretion by MDCK (I) cells was the same or slightly less than P-gp overexpressing cells, as shown in table 6-3.

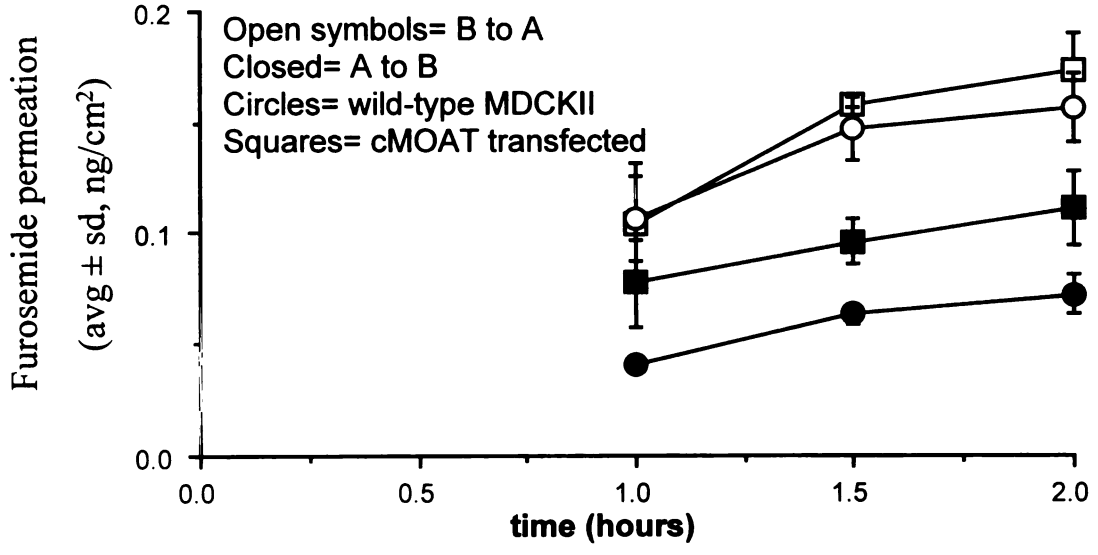


Figure 6-6: Permeation of furosemide (30 μM) across MDCKII wild-type and MRP2 transfected cells.

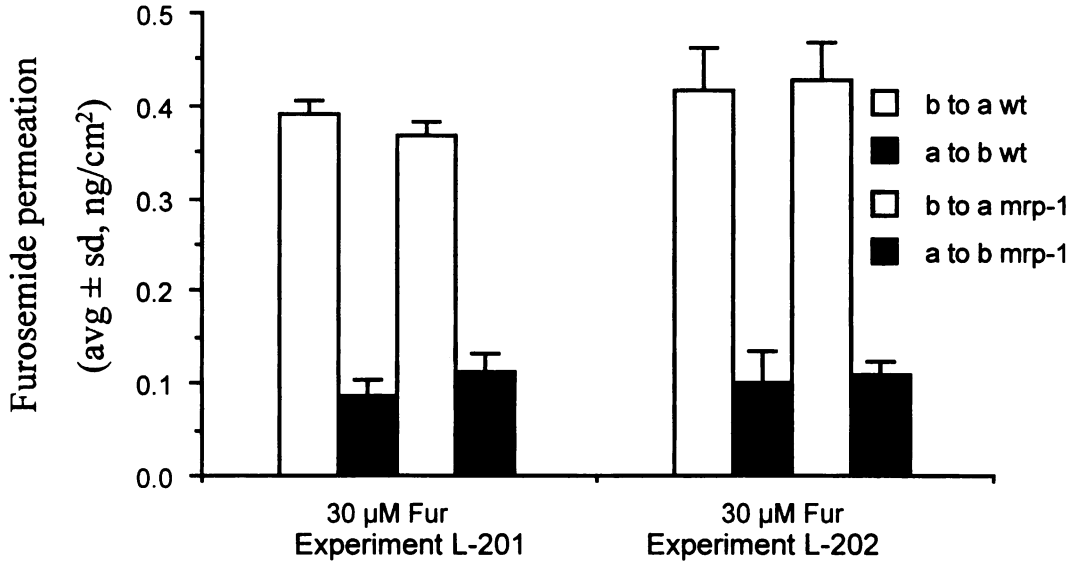


Figure 6-7: Permeation of furosemide (30 μM) across LLC-PK1 wild-type and MRP1 transfected cells.

Table 6-3: Net secretion of furosemide by endogenous MDCK tranporters.

	Net furosemide secretion (B to A/A to B)	
Cell line	20 μM	50 μM
MDCK (strain I)	3.2	3.4
MDR1 transfected	3.3	2.3

Furosemide was tested as an inhibitor of cyclosporine (5 μM) secretion by MDR1-MDCK cells. Overall net secretion of cyclosporine (3.0-fold) observed after two hours was decreased by treatment with 100 μM of either verapamil (1.2-fold) or furosemide (1.5-fold), as shown in fig. 6-8, but with both inhibitors significant inhibition was only observed in the A to B direction.

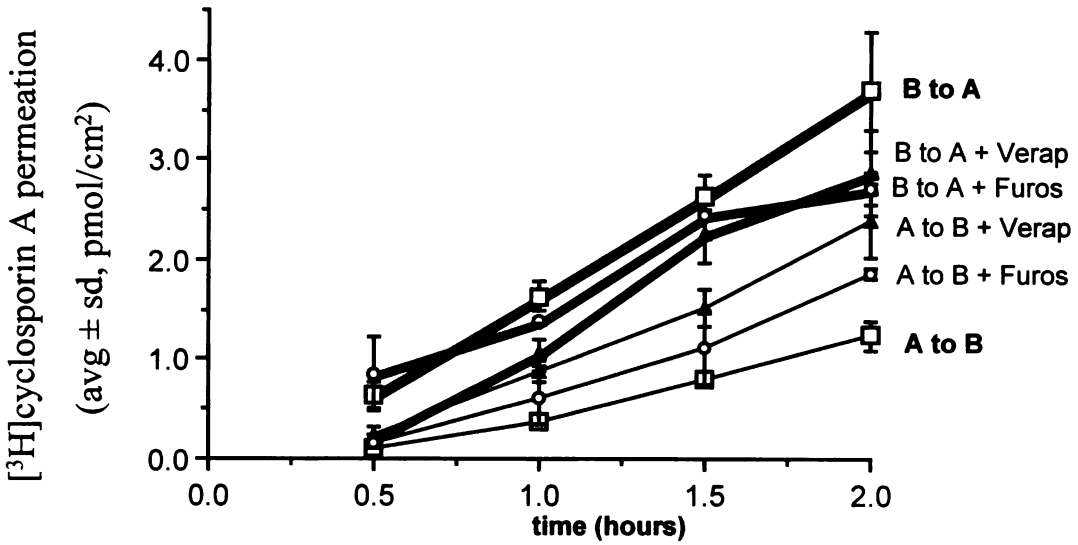


Figure 6-8: Effect 100 μM inhibitors, verapamil (Verap) and furosemide (Furos), on cyclosporine (5 μM) permeation across MDR1-MDCK cells.

6.4 Discussion

LY permeation was found to be highly variable. The compound itself is unstable in the presence of light and the analysis, which uses a fluorescent plate reader, is performed very near the level of quantitation. Despite these limitations, this method has been useful for assessing holes which result in permeability increases of an order of magnitude or greater. TEER measurements (obtained prior to experiment) also have great utility for detecting potential problems when large differences are seen. TEER values can be measured over a broad range of values, eg. 200-300 or 600-900, without seeing any correlations with permeation.

Even the paracellular marker LY behaved differently as shown by comparing tables 6-1 and 6-2. MDCKII cells could be considered more leaky than LLC-PK1 cells based on

TEER values, but less leaky to LY permeation. Similarly, differences in these two measurements between the transfected cell lines and their respective wild-types are not easily explained. Transfected MRP2 cells were leakier to LY, but TEER values -which were already at baseline levels- were constant and MRP1 transfects showed large TEER variation with no change in LY permeation relative to their respective wild-types. The most likely explanation for these differences is due to the fact that some LY permeation can occur transcellularly. The relative contribution of each route to the overall permeation of LY could vary between cell types. In addition, there is some very recent evidence that LY can be secreted in a probenecid-sensitive manner (Masereew *et al.*, 1999) and may be an unsuitable paracellular marker in certain cell lines.

Vinblastine was tested as a control substrate of MRP2 since its overexpression in MDCKII cells has been shown to result in increased secretion (Evers *et al.*, 1998). We were unable to duplicate this result as greater overall permeation was seen in both directions for the overexpressing cell line and net secretion actually went down from 14- to 8-fold (fig. 6-1). Endogenous P-gp is known to contribute to vinblastine secretion in these cells which is consistant with the inhibition in MDCKII cells observed with cyclosporine, but not sulfinpyrazone (fig. 6-2). Sulfinpyrazone, which did not inhibit the MRP2 mediated secretion of DNP-SG (Evers *et al.*, 1998), was found to decrease secretion of vinblastine in these cells, suggesting that some substrates may be sensitive to sulfinpyrazone inhibition and while others are not.

Since MRP1 has been shown to be polarized on the basolateral surface of LLC-PK1 cells upon overexpression (Evers *et al.*, 1998), absorptive transport is favored. Although Evers and co-workers (1996) reported that vinblastine efflux by MRP1 is not detectable above baseline secretion, we tested it on this cell line for lack of a better control. Permeation of vinblastine by MRP-1 transfected cells was slightly greater in the B to A direction and

slightly lower in the A to B direction indicating that MRP1 is certainly not the dominant transporter (fig. 6-4). The overall net secretion observed is presumably due to endogenous P-gp, as suggested by the inhibition effect of cyclosporine observed in both wild-type and transfected cells. This P-gp mediated secretion of vinblastine was found to decrease in wild-type LLC-PK1 cells with the addition of cyclosporine (not shown). A similar decrease with cyclosporine addition was observed in cells transfected with MRP, whereas sulfinpyrazone caused an increase in the secretion (through blockage of MRP1 which pumps in the absorptive direction) by these cells (fig. 6-5), but not the wild-type cells.

Secretion of furosemide did not change in the manner predicted by substrates for P-gp, MRP1 or MRP2 in overexpressing cells (table 6-3 and figs. 6-6 and 6-7). Secretion in the MDCKII-cMOAT cell line was found to be lower than that in the MDCKII wild-type (1.6- vs. 2.2 fold), but the positive control vinblastine also exhibited similar results. No difference was seen between LLC-PK1 wild-type and MRP1 overexpressing cell lines for furosemide secretion. Again vinblastine secretion by these cells was not different from control, as observed by Evers *et al.* (1996) even though MRP1 expression results in resistance to vinblastine in cancer cells. Sulfinpyrazone and indomethacin treatment of these transfected cells yielded the same effects observed in the wild-type cells (data not shown).

We did not test expression levels in any of these cell lines and that could contribute to the confounding results. We did try to keep the passage level as low as practical and tested within a few passages of receipt, but knowing relative expression levels between wild-type and transfected cells would have been helpful. The major factor for furosemide could be that comparisons of transport are all made to baseline secretion in kidney derived cell lines. Renal secretion is an important route of elimination, and perhaps

endogeonous renal transporters could be masking the effects of overexpressed transporters. Furosemide secretion, observed in excised rat jejunum and cultured MDCK (strains I and II), LLC-PK1 and Caco-2 cells, is not exclusively the result of any of the well characterized efflux transporters (P-gp, MRP1 or MRP2).

Chapter 7

The Effect of Oral Indomethacin on the Bioavailability of Furosemide in Healthy Volunteers

7.1 Introduction

Understanding the mechanisms underlying poor or variable oral bioavailability of drugs would help pharmacologists and drug researchers develop new and more dependable drug delivery systems. One biochemical mechanism thought to be important in altering bioavailability is the p-glycoprotein system, an ATP-dependent drug efflux system found in many tissues that pumps drugs out of the cell (Benet *et al.*, 1996). The Multi-drug Resistance-associated Protein (MRP1) is another efflux transporter known to be present in the small intestine (Kruh *et al.*,1995). P-Glycoprotein has been found to alter the bioavailability of drugs given orally (Benet *et al.* 1996; Spaareboom *et al.* 1997), and MRP1 or one of the other members of that transporter family (described in Chapter 1) could affect drugs in a similar manner.

Furosemide is a renally eliminated diuretic with poor and variable oral bioavailability. Drug interactions have been observed between intravenous furosemide and other renally eliminated compounds administered orally, such as probenecid (Smith *et al.*, 1980a) and indomethacin (Smith *et al.*, 1979; Chennavasin *et al.*, 1980), resulting in decreased renal clearance and increased plasma concentrations of furosemide. This pharmacokinetic interaction is believed to occur due to competition for a common secretory transporter

(OAT) in the kidney proximal tubule. As described in Chapter 4, the permeation of furosemide across both the excised rat jejunum, and Caco-2 monolayer *in vitro* models, is greatest in the direction of secretion suggesting that intestinal secretion may occur as well. Since this directional dependence observed *in vitro* was subject to inhibition by indomethacin, we tested the effect of indomethacin on furosemide bioavailability. Furosemide is poorly absorbed and requires a larger oral dose than when given intravenously (*iv*). There is no need to adjust the dose of furosemide when dosing with indomethacin, because indomethacin decreases the effect of the diuretic, despite an increase in plasma levels.

7.2 Materials and methods

7.2.1 Materials

All dispensed medications and placebos were obtained through the UCSF-Stanford Health Care In-patient Pharmacy at the University of California, San Francisco, Moffit Hospital. Furosemide (Lasix®) was given either as an injectible solution (40 mg *iv* by intravenous infusion over a three minute interval) or orally (*po*) as two 40 mg tablets. Indomethacin (Indocin®) was given as two 50 mg tablets *po*.

7.2.2 Human studies

The study design and protocol were approved by both the UCSF Committee on Human Research (CHR) and the General Clinical Research Center (GCRC) review boards. All subjects signed consent forms, and received monetary compensation for their participation. All of the volunteers in the study were male even though recruitment was not based on gender. The nine male subjects in our clinical study were 25 to 39 years of age and weighed from 51 to 89 kg. The individual subject details are given in table 7-1.

The participants were randomized into one of four groups of treatment sequences (A through D). All subjects had normal physical, and blood and urine screening results, testing negative for the HIV virus and drugs of abuse.

Table 7-1: Participants in research study.

Subject	Ethnicity	Group	Sequence	Age (yr)	Ht (cm)	Wt (kg)
1	Caucasian	3	CDAB	35	177	73.8
2	Caucasian	3	CDAB	38	173	73.7
3	Caucasian	1	ABCD	34	175	70.3
4	Caucasian	4	DCBA	28	180	80.5
5	Caucasian	4	DCBA	27	185	75.1
6	Caucasian	1	ABCD	39	178.5	88.2
7	Hispanic	2	BADC	36	168	51.4
8	Indian	2	BADC	25	165	66.5
10*	Hispanic	3	CDAB	29	186	88.7

*Note that subject 9 withdrew voluntarily.

On the evening prior to each of the four study days (10 hours prior to furosemide dosing), and again 30 minutes before dosing, the subjects took two tablets *po* which contained either placebo (control) or indomethacin, depending on their treatment group. Subjects were admitted as outpatients for 13-hour visits on each of the four study days and were asked to return an overnight urine collection, the following mornings. Each subject received all four of the following treatments in one of the four sequences shown in table 7-1.

A. 40 mg *iv* furosemide with placebo pre-treatment.

B. 80 mg *po* furosemide with placebo pre-treatment.

C. 40 mg *iv* furosemide with indomethacin pretreatment.

D. 80 mg *po* furosemide with indomethacin pretreatment.

Phosphoric acid (0.5 ml per container) was added to each urine collection jar as a preservative prior to the study. Voided urine was collected at 11 intervals (0-0.5, 0.5-1, 1-1.5, 1.5-2, 2-3, 3-4, 4-5, 5-6, 6-8, 8-12, and 12-24 hours). Approximately 5 ml of each acidified urine sample was stored at -25 °C for analysis, and the remaining urine was pooled with previous samples (and combined for all patients) for subsequent extraction of furosemide-glucuronide (F-G) metabolite. Whole blood was collected in heparinized tubes, placed on ice and centrifuged at 0 °C. Plasma was collected in duplicates for each of the 15 time points and then stored at -25 °C. Sampling times varied between the *iv* (0, 5, 10, 20, 40, 60, 90 and 120 min) and *po* (0, 10, 20, 40, 60, 80, 100 and 120 min) treatments over the first two hours to better determine C_{max}. These collections were followed by four hourly collections and three collections every 2 hours for the remainder of the study (180, 240, 300, 360, 480, 600, and 720 minutes) regardless of route of administration.

7.2.3 Data analysis

Data was analyzed using WinNonlin-Pro™ 2.0 (Pharsight Corporation, Cary, NC) to determine area under the concentration *vs.* time curve extrapolated to time infinity (AUC), maximum concentration (C_{max}), and terminal half-life ($t^{1}/_{2}$). Total clearances (CL) for the *iv* treatments were calculated by dividing Dose by the AUC (CL= D/AUC). Bioavailability (F) for the placebo (control) and indomethacin pre-treatments was obtained by the following formula:

$$F = AUC_{po} \times D_{iv} / AUC_{iv} \times D_{po}$$

7.2.4 Analytical methods

Carprofen was used as an internal standard for indomethacin, and iminostilbene was the internal standard for furosemide and F-G. Analysis was by HPLC with fluorescent detection at a flow rate of 1.0 ml/min. Furosemide and F-G methods were updated from

those previously published (Smith *et al.*, 1980b). Protein was precipitated and removed by the addition of acetonitrile to plasma samples (under acidic conditions for furosemide and F-G) prior to analysis.

A fluorescent photo irradiation product of indomethacin was cleaved by a pre-column photo irradiation pen (Fisher, Pittsburgh, PA) and analyzed with excitation and emission of 370 and 470 nm using a C8 column (4.6 mm x 150 cm x 5 μm particle size). Indomethacin mobile phase contained 53% acetonitrile and 47% water with 0.1% (v/v) each of phosphoric acid and hydrogen peroxide. Mobile phase for furosemide and F-G contained 30% acetonitrile and 70% water with 0.2% (v/v) of phosphoric acid. Furosemide and F-G were analyzed using an excitation and emission of 345 and 405 nm and a C18 column (4.6 mm x 150 cm x 5 μm particle size). Standards for F-G were prepared by isolating this metabolite from pooled urine of all subjects. Urine was concentrated by thawing 50% of the volume and discarding the solid ice block. Urine concentrate was further purified using an open C18 column.

7.3 Results

This chapter contains preliminary plasma data for the first three subjects (2, 3 and 8) analyzed. The plasma concentration of furosemide *vs.* time profiles after *iv* administration for each subject (with and without indomethacin pretreatment) are plotted with linear and semilog scales in figs. 7-1 and 7-2, respectively. Indomethacin pretreatment resulted in higher plasma levels in all three subjects. The individual results upon *po* administration of furosemide are shown with linear and semilog scales in figs. 7-3 and 7-4, respectively. The effects of indomethacin on orally administered furosemide are less pronounced than for intravenous administration, and it is difficult to see any change between these two treatment groups.

In order to better assess the effect of indomethacin pretreatment on furosemide pharmacokinetics, a number of parameters were calculated, as shown in table 7-2. Due to large variation and this small preliminary sample size, no significant differences in any parameter were seen with indomethacin pretreatment. Previously we showed that indomethacin pretreatment caused an increase in C_{max} and AUC and a decrease in clearance (Smith *et al.*, 1979), and these same changes were seen for these three subjects.

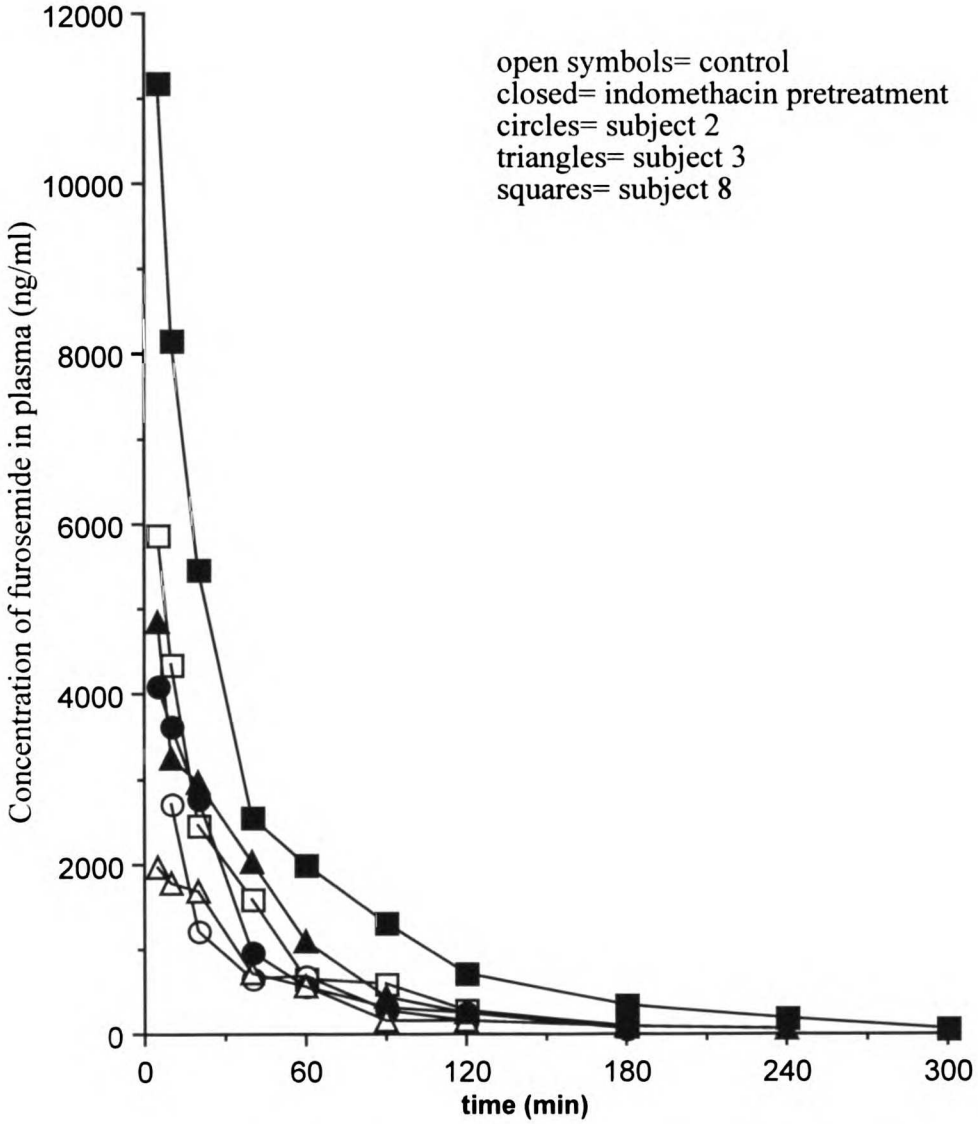


Figure 7-1: The effect of indomethacin pretreatment on intravenous furosemide (linear scale).

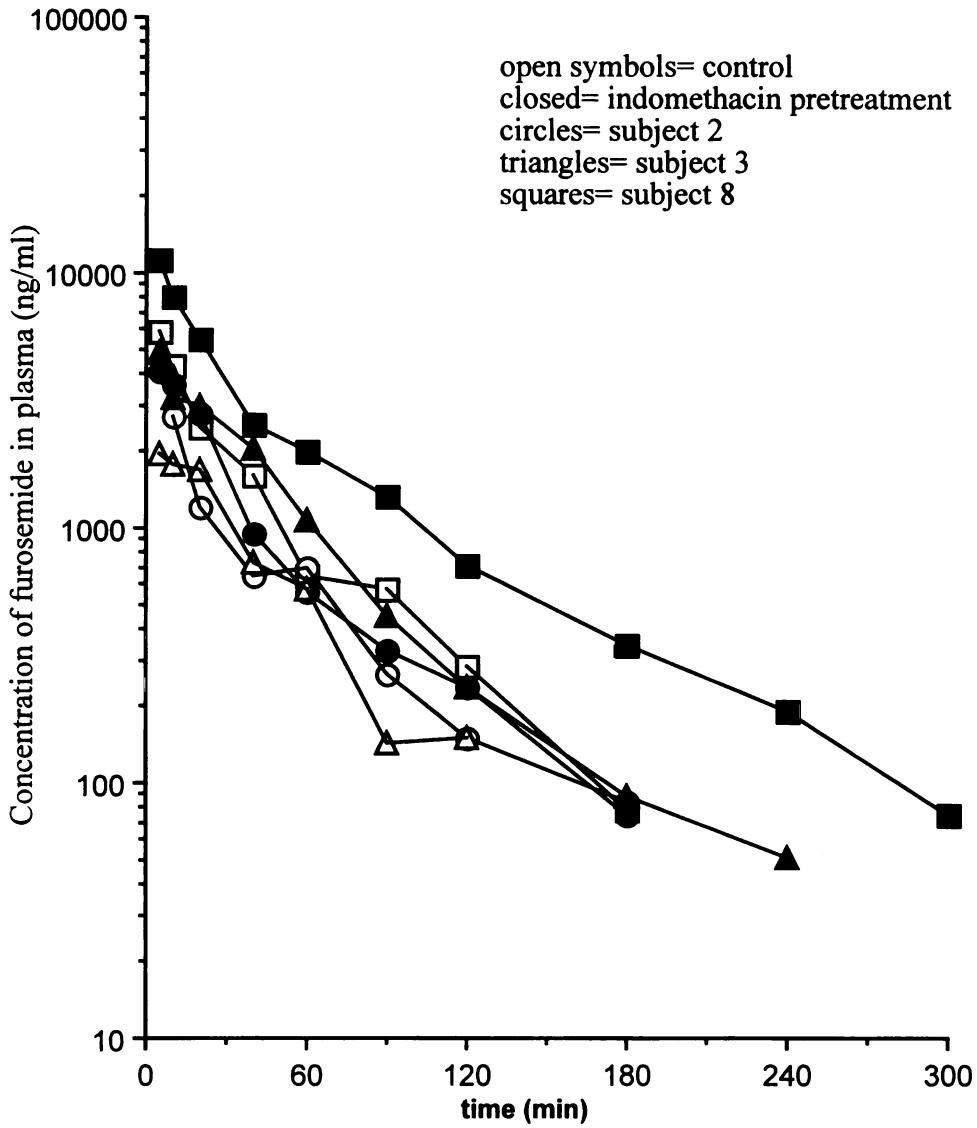


Figure 7-2: The effect of indomethacin pretreatment on intravenous furosemide (semi-log scale).

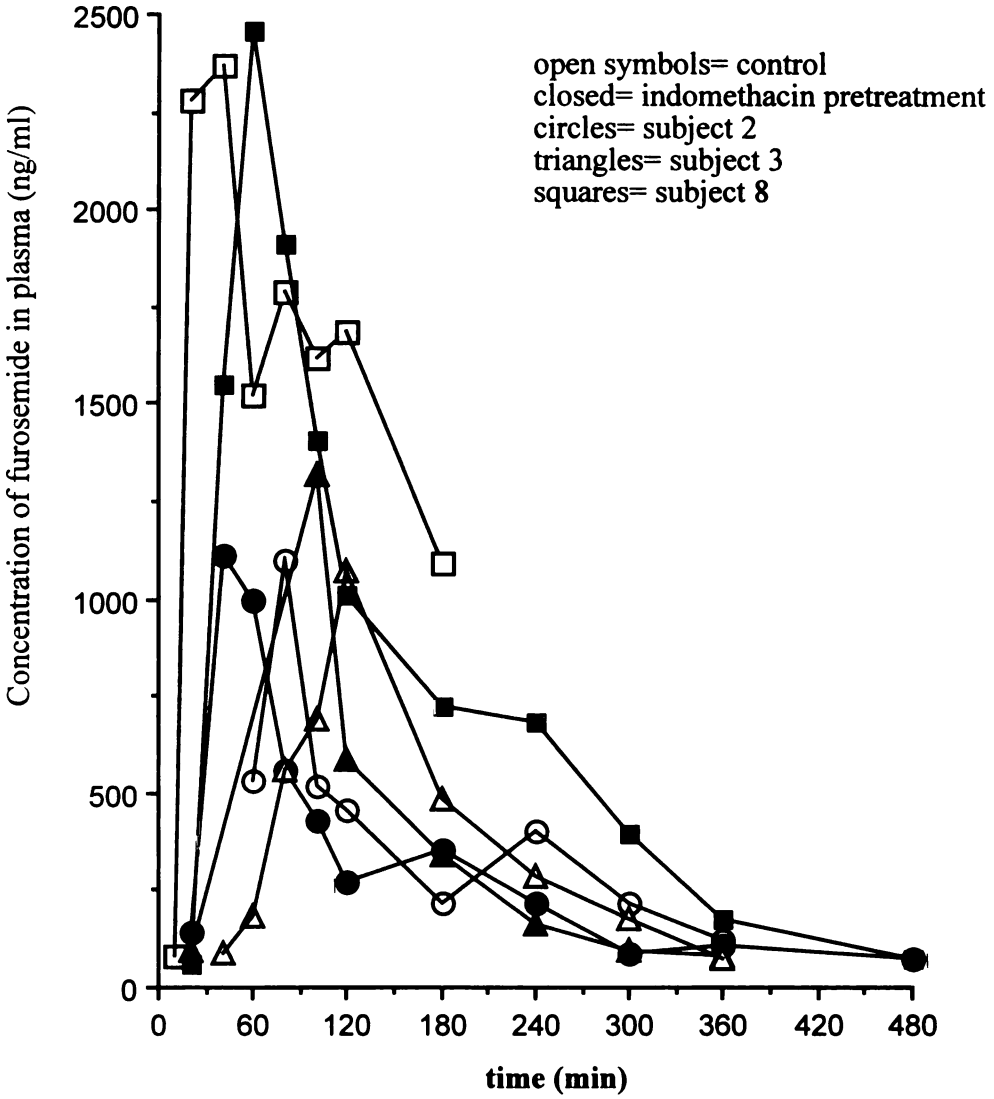


Figure 7-3: The effect of indomethacin pretreatment on oral furosemide (linear scale).

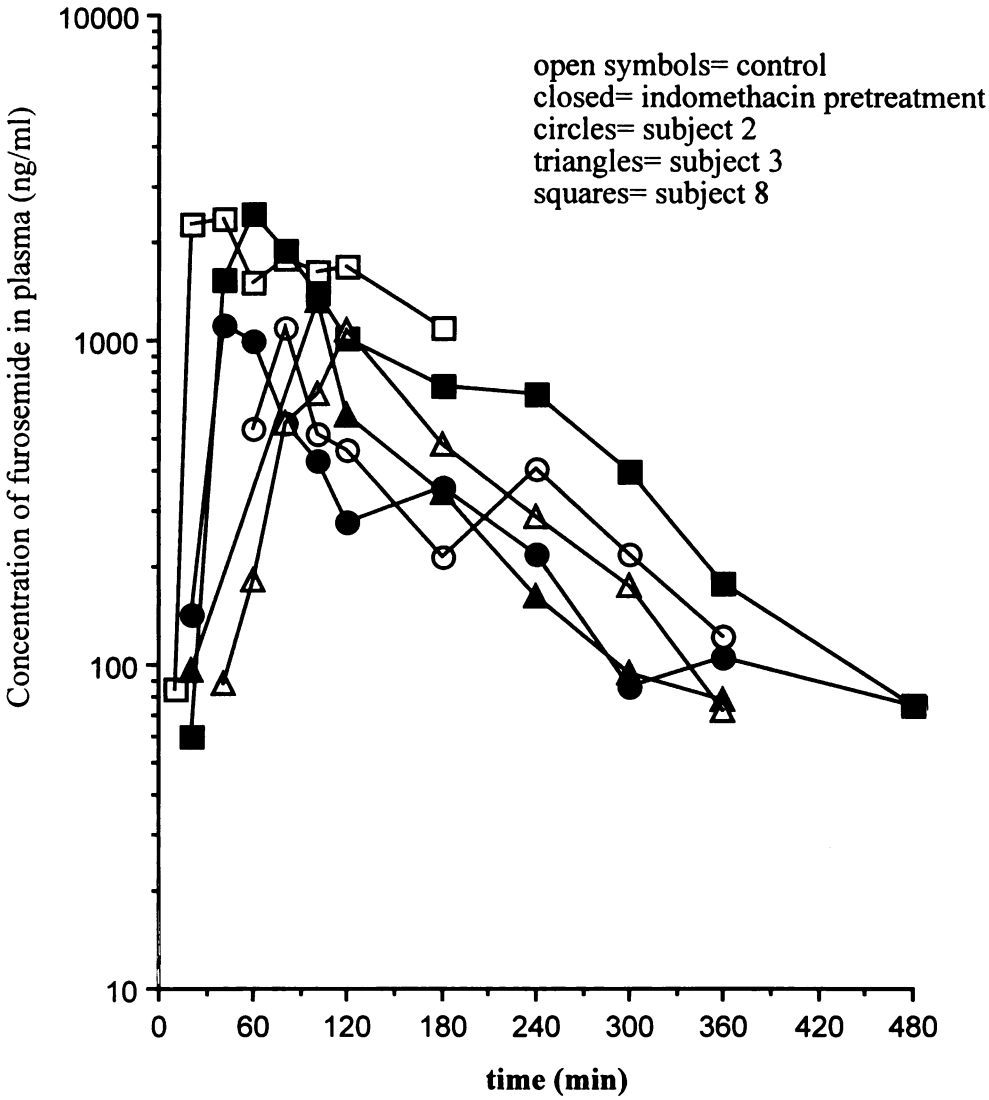


Figure 7-4: The effect of indomethacin pretreatment on oral furosemide (semi-log scale).

Table 7-2: Pharmacokinetic parameters.

Subject no.	$t^{1}/_{2}$ (min)	C_{max} (μg/ml)	AUC (μg•min/ml)	percent extrapolated	CL* (ml/min)
Treatment A					
2	40.9	2.7	86.0	5.5	465.1
3	27.4	2.0	83.8	6.7	477.1
8	31.3	5.9	179.8	1.9	222.5
Mean	33.2	3.5	116.5	4.7	388.2
SD	7.0	2.1	54.8	2.5	143.6
Treatment B					
2	69.9	1.1	124.6	9.9	642.3
3	65.2	1.1	135.7	5.0	589.3
8	139.8	2.4	499.1	44.0	160.3
Mean	91.6	1.5	253.1	19.6	464.0
SD	41.8	0.7	213.1	21.2	264.3
Treatment C					
2	39.7	4.1	145.5	2.9	275.0
3	34.7	4.9	192.0	1.3	208.3
8	51.6	11.2	406.9	1.3	98.3
Mean	42.0	6.7	248.1	1.8	193.9
SD	8.7	3.9	139.5	0.9	89.2
Treatment D					
2	246.0	1.1	153.8	12.4	520.1
3	78.8	1.3	138.3	6.5	578.4
8	74.3	2.5	321.1	2.5	249.2
Mean	133.0	1.6	204.4	7.1	449.2
SD	97.9	0.7	101.3	5.0	175.7

* Clearance values for treatments B and D are "oral clearances", i.e. CL/F.

The bioavailability of furosemide, with and without indomethacin pretreatment, is presented in table 7-3. Again variation was large and significant differences between treatments could not be seen, but the observed trend showed that indomethacin decreased the bioavailability in all three subjects.

Table 7-3: Effect of indomethacin on furosemide bioavailability.

Subject no.	Bioavailability	
	Control	Indomethacin pretreated
2	0.72	0.53
3	0.81	0.36
8	1.39	0.39
Mean	0.97	0.43
SD	0.36	0.09

7.4 Discussion

A number of surprising results are seen in this preliminary data. Clearance values were on average three times higher, and half-life values were one-third lower (table 7-2) than we had previously seen for intravenous furosemide (Smith *et al.*, 1980). In addition, the bioavailability was also greater than in our earlier study (table 7-3).

Clearance values for subject 8 were closer to the value we previously observed, but this and most other parameters varied considerably from the other two subjects. From Table 7-1, we can see that weight and height were similar between subjects, but that subject 8 is younger than the others (25 *vs.* 34 and 38). In addition, ethnic differences may exist as subjects 2 and 3 are caucasian males, whereas subject 8 is an male of Indian decent. The impossibly high bioavailability (1.39) of subject 8 (table 7-3) is largely due to the error in extrapolating 44% of the AUC as a result of that subjects long half life (table 7-2). We will test this value for the terminal elimination of furosemide in this subject from the urine data.

The biggest surprise was that bioavailability did not increase in the presence of indomethacin and it even decreased. This decrease was due to the fact that indomethacin

actually affected the furosemide *iv* dose differently than the *po* dose, even though the same indomethacin protocol was used in both cases. As shown in Chapter 4 (Flanagan and Benet, 1999), indomethacin blocks furosemide intestinal secretion *in vitro*, but this was apparently not the case *in vivo*.

Chapter 8

Conclusions and Perspectives

This research was initiated as a literature screening project in an effort to find a novel class of P-glycoprotein substrates, and instead has lead seemingly to a novel or uncharacterized transporter. Diuretics have long been known as a class of compounds having low bioavailability and were considered as possible substrates of P-gp for a number of reasons. Most diuretics are substrates for renal secretory transporters. Some of these transporters could be present in both the kidneys and small intestine and contribute to the overall poor oral bioavailability. In addition, there is a certain amount of overlap between substrates of different transporters. Although not tested rigorously, it seems that for a given xenobiotic, transport by any protein is much more likely to occur if that compound is already known to be transported by some other transporter. In addition to the literature that suggests that secretion may occur for some diuretics (cited in Chapter 1), there were reports of drug interactions with other P-gp substrates such as digoxin and cyclosporine. The possibility that diuretics may be secreted in the small intestine seemed worthy of testing and has been studied using two model compounds, amiloride and furosemide, in two in vitro absorption models.

Both amiloride and furosemide showed net secretion from excised rat jejunum and Caco-2 cells. Amiloride secretion from Caco-2 cells did not demonstrate any temperature or

chemical inhibition. In addition, the flux of amiloride across Caco-2 cells increased linearly over a wide range of concentration values (Chapter 3). Amiloride may be absorbed via the paracellular route, as indicated by sensitivity to EGTA-treatment. Recent studies have shown that some drugs, such as ranitidine and verapamil, are capable of modifying their transport across this route and the effect can vary based on which side of the membrane the drug is on. The possibility exists that amiloride is effecting its own permeability by modulating tight junctions in a concentration dependent manner, such that a bigger effect is seen when the donor solution (highest concentration) is on the side where the biochemical interaction is occurring. The possibility that a general artifact exists, in that the small intestine is just more permeable to bases in the outward direction, cannot be ignored.

Unlike amiloride, furosemide transport was found to be dependent on both temperature and concentration in Caco-2 cells, suggesting secretion in a carrier-mediated manner (Chapters 4 and 5). Active transport of furosemide was confirmed by demonstrating that secretion was energy dependent. Using chemical inhibitors to identify a specific inhibitor responsible for the transport of furosemide proved to be an imprecise approach, due to the overlap of substrates. Inhibition of furosemide secretion from Caco-2 cells by indomethacin, probenecid and sulfinpyrazone suggested that OAT or an MRP could be involved. Similarly, inhibition by cyclosporine and vinblastine, substrates of both P-gp and MRP, failed to identify a specific transporter. It seemed likely that MRP could play a role in the secretion of furosemide, since both groups of inhibitors interacted with MRP, but could not be confirmed without a specific inhibitor.

We obtained epithelial cell lines that other researchers had transfected with specific human transporters in an attempt to better characterize the secretion of furosemide. Transport studies were conducted using P-gp, MRP1 and MRP2 transfected cell lines (Chapter 6). Comparisons between furosemide secretion observed on these cell lines did not vary from the non-transfected wild-type cells. From these data, it seems that neither of those three transporters participates in the secretion of furosemide. The possibility does exist however, that the endogenous canine or porcine transporters found in those renal cell lines are masking the secretion by the transfected human transporters. Use of a knock-out animal would provide a more definitive answer. At this point the identity of the transport protein responsible for the secretion of furosemide is a mystery. Likely candidates include members of the MRP or OAT families that have yet to be fully characterized (or even discovered). It is unlikely that P-gp would transport furosemide, given the negative charge on the molecule at physiological pH.

Indomethacin caused a marked decrease in the secretion of furosemide from Caco-2 cells (Chapter 4). Orally administered indomethacin has long been known to have a pharmacokinetic drug interaction with intravenous furosemide. Indomethacin and other organic anions, such as probenecid, block renal secretion (decreasing renal clearance) thereby increasing furosemide plasma concentrations. A clinical study was initiated to determine if indomethacin could increase the bioavailability of furosemide, which would suggest inhibition of intestinal secretion. However, preliminary results from three of nine subjects showed that indomethacin pretreatment may be decreasing the bioavailability (Chapter 7). It remains to be seen if this trend will hold, but it certainly complicates our understanding of the secretory processes in the gut as they relate to furosemide.

Other diuretics, bumetanide, chorothiazide and triamterene, appeared to be secreted from Caco-2 cells (Chapter 5). It remains to be seen if this secretion is due to a transport protein, as is the case for furosemide, or like amiloride- it could merely be some architectural artifact. Again, it is most important to determine what effect this will have *in vivo*.

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