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

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Purpose. To determine if intestinal secretion occurs for the poorly bioavailable diuretic, furosemide.
Methods. Jejunal segments of male Sprague-Dawley rats were mounted on diffusion chambers, and the permeation of furosemide was measured across the excised tissue in both directions. Studies were repeated using cultured epithelia from adenocarcinoma cells (Caco-2) grown on filter inserts mounted in 6-well plates. Temperature-dependence and chemical inhibition by indomethacin was also tested using the cell culture model.
Results. Net secretion from rat intestine of over 3-fold was observed for 20 μM furosemide. Net secretion of furosemide by Caco-2 cells was over 300% greater than for intestinal segments (10-fold *vs.* 3-fold). For both models, a decrease in furosemide transport in the direction of secretion was observed in the presence of indomethacin (100 μM), although only results using the Caco-2 cells showed an increase in the absorptive transport. Furosemide secretion from Caco-2 cells decreased with decrease in temperature from 37°C to 4°C, suggesting a carrier-mediated process.
Conclusions. Furosemide appears to be secreted in the small intestine. These preliminary results indicate that furosemide bioavailability may be limited by an intestinal transporter.

KEY WORDS: intestinal transport; rat jejunum; Caco-2 cells; furosemide; indomethacin.

INTRODUCTION

Furosemide is a potent and widely used diuretic with highly variable and poor bioavailability, 11 to 90% (1). The pharmacokinetics and pharmacodynamics of this drug have been extensively studied and reviewed (2), yet the cause of this low bioavailability remains unclear. Bioavailability from oral solution and tablet formulations do not vary, suggesting that solubility and dissolution rates are not important parameters (3). Gastric elimination by acid hydrolysis has also been ruled out as a significant cause for the drug's poor bioavailability (4). Perhaps the best explanation is that furosemide may be secreted by the intestine. This has been widely speculated (5–7), but never demonstrated.

In 1986, Valentine *et al.* (7) showed that half of the elimination of an intravenous furosemide dose occurred non-renally and non-metabolically, but that experimentally 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 (8). A recent study, using isolated rabbit kidneys has demonstrated this hypothesis to be valid (9). We believe that intestinal 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 (10) and for digoxin in mice (11).

Recent studies in our laboratory and others have shown that the small intestine functions as a major organ of elimination (10–12). Efflux transporters, such as P-glycoprotein (13,14), 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 (14).

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 (1). 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.

MATERIALS AND METHODS

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).

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 Kreb's-Ringer Bicarbonate solution (KRBS), 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

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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 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.

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 for 30 min in MEM without supplements (transport media), and tested with 1.5 ml in the apical chamber and 2.5 ml in the basolateral chamber. 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 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.

Analytical Methods

Samples in media or buffer were analyzed by reverse-phase chromatography and fluorescent detection using previously published methods (15), without extraction. Briefly, furosemide analysis was performed with a 4.6 mm × 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.

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 (16), 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. 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. 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.

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 them to be a highly useful model for absorption studies (17,18) having few limitations (19). 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. 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. 2 and 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. 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.

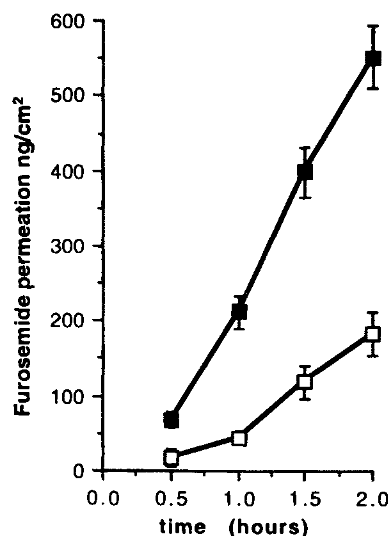

Fig. 1. Permeation of furosemide (20 μM) through excised male Sprague-Dawley rat jejunum. Open squares for mucosal-to-serosal, and closed squares for serosal-to-mucosal directions. Each point represents the mean ± SE of n = 10, from N = 6 rats.

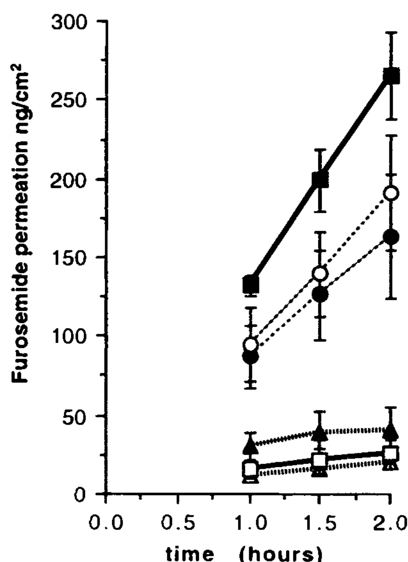

Fig. 2. Permeation of furosemide (30 μM) through Caco-2 epithelia is subject to temperature and chemical inhibition. Open symbols for mucosal-to-serosal, and closed for serosal-to-mucosal directions. Controls (37°C) are shown as squares, with triangles for reduced temperature (4°C). Circles are used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

Transport in the A-to-B direction was not significantly different at 37° or 4°C.

Figure 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 I, but there was no effect on A-to-B transport for these compounds.

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 (data to be published elsewhere) have shown that secretion of furosemide is also saturable.

Table I. 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

The apparent secretion of furosemide observed 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. 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 (20), 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 (21). 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 (22), and phenol red in excised rat jejunum (23). These authors were unable to implicate a specific transporter, although Collington *et al.* in 1992 (22) 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 (24).

At least six different MRP's have been identified, but only MRP-1 has been demonstrated to convey multidrug resistance (25). In addition to indomethacin sensitivity, MRP-1 has been shown to be inhibited by probenecid, vinblastine, and cyclosporine (whose effects on the intestinal secretion of furosemide are shown in Table I) in cancer lines transfected with or overexpressing MRP, but not p-glycoprotein (26). 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 (27); if a similar location is found in human small intestine then MRP-1 cannot be responsible for furosemide's apparent secretion. The canalicular multispecific organic anion transporter (cMOAT, a.k.a. MRP-2) which may be responsible for cisplatin resistance (25), 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 (28). Definitive studies are likely to require the use of molecular biology technologies such as transgenic cell lines (27,28), and or "knock-out" mice (11).

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.

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