Profound Effect of Plasma Protein Binding on the Polarized Transport of Furosemide and Verapamil in the Caco-2 Model

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INTRODUCTION

Caco-2 cell monolayers have been widely used as a very valuable *in vitro* model to study intestinal permeability of drugs for correlation with or prediction of human absorption, to rapidly screen a large number of potential drug candidates for intestinal absorbability, and to study kinetics and mechanisms of intestinal absorption, exsorption and metabolism (1-8). In most studies reported to date, aqueous buffered solutions were used as media in the basolateral compartment for measurement of the apical-to-basolateral (A→B) absorptive permeability or transport clearance (9,10), basolateral-to-apical (B→A) efflux (exsorption) permeability or clearance (9,10), and net secretion ratio (B→A/A→B) involving efflux transporters such as P-glycoprotein (P-gp). Since mesenteric blood perfuses enterocytes near the basolateral side and the plasma protein binding may theoretically affect drug absorption and exsorption *in vivo,* it was decided to study the effect of using human plasma as a basolateral medium on the bidirectional transport of two efflux transporter substrates, furosemide (6) and verapamil (11), both of them being extensively bound (99% and 90%, respectively) to plasma proteins (12).

METHODS

Furosemide, mannitol, and verapamil were purchased from Sigma Chemical Co. (St. Louis, MO). Human plasma was obtained from the hospital blood bank of the University of Illinois at Chicago with sodium citrate as anticoagulant. Caco-2 cells (passage number 18, American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Grand Island, NY) supplemented with 10% fetal calf serum, penicillin (100 UI/ml), and streptomycin (10 μg/ml), and cells were grown in a humidified atmosphere of 5% CO_2 at 37°C. Cells were passed when they reached approximately 80% confluency. For all transport studies, Caco-2 cells were seeded on top of Transwell® inserts (polycarbonate membrane; 24.5 mm diameter, 4.71 cm^2 surface area, and 0.4 μm pore size, Corning Costar Corp., Cambridge, MA) in 6-well plates at a density of 5.0×10^5 cells/ml. Culture medium of 2.5 and 1.5 ml were added to the basolateral and apical compartment, respectively. Medium was changed every other day for two weeks and daily thereafter. Transport experiments were performed between 18 to 20 post-seeding days. Culture medium was removed and cell monolayers were washed three times with the aqueous transport medium, which was Hank's Balanced Salt Solution with 25 mM HEPES at pH 7.4. Transepithelial electrical resistance (TEER) was measured in transport medium with Epithelial VoltOhummeter® (World Precision Instruments, Sarasota, FL). Average TEER value was 240 ± 10 Ohm cm^2 indicating the integrity of cell monolayers. Also, the bidirectional flux of mannitol was virtually identical whether the aqueous buffer solution or human plasma was used as medium in the basolateral compartment, indicating a lack of effect of human plasma on the integrity of tight junctions in the present study.

Transepithelial transport rates were measured for mannitol, furosemide, and verapamil at final concentrations of 200, 50, 5 μM, respectively. For A→B transport studies, 1.5 ml of the aqueous transport medium containing the test compound was added to the apical chamber and 2.5 ml of either aqueous transport medium or human plasma were added to the basolateral chamber. For B→A transport studies, 1.5 ml of the aqueous transport medium was added to the apical chamber and 2.5 ml of aqueous transport medium or human plasma containing the test compound was added to the basolateral chamber. Transport studies were conducted at 37°C with 60-rpm oscillation in a water-bath shaker. On termination, samples were collected from both sides of the cell monolayer and analyzed by HPLC for furosemide (13) and verapamil (14) and by LC/MS for mannitol. To measure adsorption of drugs to polycarbonate membrane and Transwell®, directional transport rates were measured without the cell monolayers. No adsorption to the device was found.

The transport clearance per unit surface area (CL_{tp}) or effective permeability (P_{eff}) was calculated using the following equation (both CL_{tp} and P_{eff} have identical absolute value and unit but have entirely different physical meanings and derivations, references 9 and 10; CL_{tp} are preferred by us from a scientific point of view):

$$CL_{tp} = \frac{\Delta C}{\Delta t} \cdot \frac{V}{C_0} \cdot \frac{1}{A}$$

where A is the surface area of Caco-2 monolayer (4.71cm^2), $\triangle C/\triangle t$ is the change in drug concentration in the receiver solution over a period of time, $\triangle t$, V is the volume of the solution in the receiving compartment, and C_0 is the initial drug concentration in the donor compartment. Sink conditions in the receiving compartment were maintained during the study since only about 2 % of furosemide and 10 % of verapamil were transported during the study period. The data were expressed as the mean ± standard deviation (S.D.); except for the A→B study for verapamil three studies were performed. Student *t*-test was used for the statistical evalua-

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tion of the difference of mean at 95% confidence interval. Net apical secretion ratio was calculated by the B→A CL_{tp} divided by A→B CL_{tp} (6).

RESULTS AND DISCUSSION

The results of our polarized transport study of furosemide and verapamil using aqueous buffer or human plasma as a medium in the basolateral compartment are summarized in Table I and depicted in Fig. I. The present findings and potential significance are elaborated below.

Apical-to-Basolateral (A→B) Transport

Compared to aqueous buffer, use of human plasma as a basolateral medium increased the A→B CL_{tp} or P_{eff} by 7.6-fold and 4.2-fold for furosemide and verapamil, respectively. These results suggest that plasma protein binding may have a dramatic effect on the absorptive transport in an *in vitro* cell-line model. Our present findings are consistent with an earlier excellent study (15) on the effect of protein binding on the absorptive transport of chlorpromazine across the MDCK cell monolayers. By using the 0.1 % albumin solution as an approximate control (only about 4% bound), use of 3% albumin (resulting in 57% bound) and 0.1% α-acid glycoprotein (resulting in about 92% bound, ref. 16) can be estimated to increase the cumulative absorptive transport (sink conditions in the basolateral compartment were maintained by frequent change of medium) at 180 min by 1.4-fold and 6.1-fold, respectively (Fig. 2). In spite of complications due to use of different drugs, cell lines and experimental conditions, a general rank-order relationship between change in absorptive transport and percent of plasma protein binding from the present and earlier (15) studies appears evident (Fig. 2). The above data suggest that the extent of plasma protein binding may influence the rate of the gastrointestinal absorption of drugs *in vivo*. These results are consistent with an earlier study (17) that the intestinal absorption of sulfadimethoxine, a highly protein-bound drug, was markedly (about 60%) reduced by intravenous coadministration of salicylic acid or phenylbutazone, both being highly protein-bound, that could reduce the protein binding of sulfadimethoxine by competitive replacement. On the other hand, the coadministration practically had no effect on the intestinal absorption of sulfanilamide that showed little binding to plasma proteins (17).

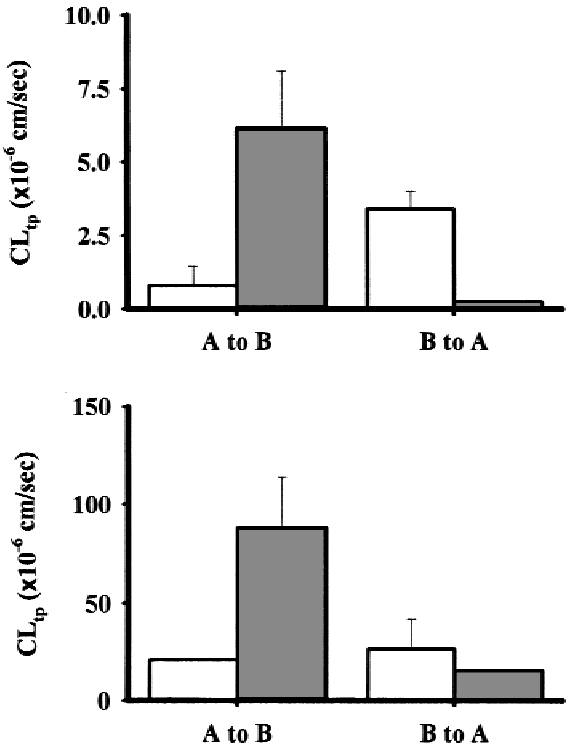

Fig. 1. Transepithelial transport clearance (CL_{tp}) of furosemide (top) and verapamil (bottom) with conventional aqueous buffer (□) or human plasma (▨) as a basolateral medium in the direction of apical to basolateral (A to B) and basolateral to apical (B to A) transport.

They are also consistent with frequent observations that intestinal absorption of water and many compounds can be markedly enhanced by increase in mesenteric blood flow rate (10, 18). The above *in vitro* and *in vivo* data may be rationalized by the hypothesis that although the basolateral medium in the present and earlier (15) studies as well as the mesenteric capillary plasma in animal studies (17) were all kept under sink conditions, the drug concentrations in unstirred water layer near basolateral membranes of the monolayers or near the wall in the vascular capillary may not be in sink conditions as commonly assumed (9,10,18). Our work is, however, different from the one published recently (19) in which no significant effects of plasma protein binding on the absorp-

Table 1. Net Apical Secretion Ratio (B→A/A→B) of Six Compounds with Basolateral Aqueous Buffer or Human Plasma Across Caco-2 Cell Monolayers and Their Unbound B→A Transport Clearances

	Net apical secretion ratio				
	Aqueous buffer	Plasma	% decrease in plasma medium	Fraction unbound in plasma[b]	Unbound CL_{tp}[c] (B→A) (10^{-6} cm/sec)
Furosemide	4.18	0.04	99	0.01	25
Oestradiol 17 β-D-glucuronide[a]	1.02	0.42	59	0.04	13.5
Propranolol[a]	0.65	0.11	83	0.1	42
Quercetin[a]	0.91	0.05	94	0.01	130
Taxol[a]	8.77	1.73	80	0.05	144
Verapamil	1.27	0.17	86	0.1	152

[a] Data were obtained from reference 19.
[b] Data were obtained from references 12 and 19.
[c] Unbound transport clearance = CL_{tp}/fraction unbound in plasma.

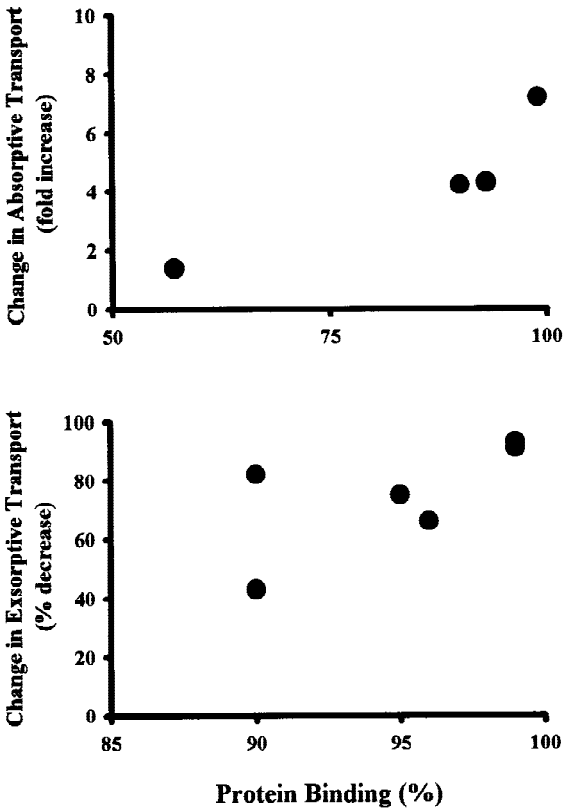

Fig. 2. Relationship between change in absorptive transport (top) or in exsorption transport (bottom) and percent of plasma protein binding of compounds. In addition to transport data of furosemide and verapamil from our results, other absorptive transport data were obtained from ref. 15 and exsorptive transport data were obtained from ref. 19.

tive transport of four high plasma protein binding compounds (propranolol, 90% bound, quercetin, 99 % bound, oestradiol β-D-glucuronide 96 % bound, and taxol, 95 % bound) were found. The reason for this difference is not known and the difference is complicated by different Caco-2 culture conditions and probably other different experimental conditions used between the two studies.

Since P_{eff} or CL_{tp} across Caco-2 cell monolayers has been commonly employed to predict or correlate with the fraction of compounds absorbed in humans (2,4,5), the results of the present studies may be potentially significant, especially for highly protein-bound compounds. In an early study on correlation between the P_{eff} in Caco-2 cells and the fraction of oral dose absorbed in humans (5), furosemide appeared to be an outlier in the correlation. Interestingly, a 7.4-fold increase in P_{eff} of this drug observed in the present study with human plasma as a basolateral medium would tend to make the drug fall near the reported correlation curve.

Basolateral-to-Apical (B→A) Transport

Compared to the aqueous buffer, use of plasma as a basolateral medium was found to have practically no effect on the B→A transport rate of mannitol. This was not unexpected since mannitol was not bound to plasma proteins, and the unbound drug concentration is the main driving force for transport across cell membranes (19). On the other hand, the mean B→A CL_{tp} was decreased by 93% for furosemide and 43% for verapamil (Fig. 1). During our study, we noticed similar results reported (19) for the four highly protein-bound compounds: the fluxes of oestradiol 17-β-D-glucuronide, taxol, propranolol and quercetin were reduced by 66, 75, 82 and 94 %, respectively. Taken together, the results of the present and earlier (19) studies generally show a rank-order relationship between the extent of plasma binding and the decrease in B→A transport (Fig. 2). Interestingly, when the B→A transport clearances were expressed in terms of unbound drug concentrations (i.e., CL_{tp} divided by fraction unbound, ref. 20), unbound transport clearances from the present and earlier (19) studies were much higher (Table I) than the transport clearances obtained using the aqueous buffer as a basolateral medium; the increases ranged from 1.8-fold for propranolol to 8.6-fold for oestradiol 17-β-D-glucuronide. These marked increases in unbound clearances are likely attributed to the more efficient active efflux at lower concentrations (i.e., less saturable at low concentrations). The above reductions in B→A transport are consistent with the earlier rabbit study (10) showing enhanced exsorption of sulfadimethoxine but not sulfanilamide following intravenous coadministration of salicylic acid or phenybutazone.

Basolateral-to-Apical / Apical-to-Basolateral Transport Ratios

Measurement of *in vitro* B→A/A→B transport ratios has been commonly employed as a valuable tool to study the role of efflux transporters such as P-gp. During the course of our study, question of potential impact of plasma protein binding in basolateral medium of the Caco-2 model on the net secretion ratio has been raised (19). The profound effect (19) of protein binding on the net secretion ratio of four compounds is also listed in Table I. Among the six compounds examined, the reductions in ratio ranged from 59 % for oestradiol 17-β-D-glucuronide to 99 % for furosemide. For furosemide and verapamil, the secretion effect or the absorption-retarding effect of the efflux transporter in the study using aqueous buffer as a basolateral medium is evident as their net secretion ratios were greater than one; our results are similar to those reported earlier (6,11). However, this ratio was reduced to only about 0.04 for furosemide and 0.17 for verapamil indicating a much less significant efflux impact under a more physiologically relevant condition employed here. The above finding may partly explain why the efflux transporter apparently has no significant limiting effect on the *in vivo* oral absorption of many drugs (21,22). For furosemide, this is demonstrated by similar bioavailability and T_{max} in humans with a doubling of dose (23,24), and by a similar fraction absorbed in rats over a 120-fold dose range (13). For verapamil, complete absorption in humans was found over a 3-fold dose range (23) indicating an apparent lack of significant effect of P-gp (21,22). In an earlier study (6), a high net secretion ratio of about 10 was postulated to account for incomplete (about 60%) absorption of furosemide in humans. High effective intestinal permeability in humans has been postulated to rationalize complete oral absorption of verapamil in humans even though it is a P-gp substrate (25,26).

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