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Research Article

Permeation of Four Oral Drugs Through Human Intestinal Mucosa

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Abstract. The pharmaceutical industry is in need of rapid and accurate methods to screen new drug leads for intestinal permeability potential in the early stages of drug discovery. Excised human jejunal mucosa was used to investigate the permeability of the small intestine to four oral drugs, using a flow-through diffusion system. The four drugs were selected as representative model compounds of drug classes 1 and 3 according to the biopharmaceutics classification system (BCS). The drugs selected were zidovudine, propranolol HCl, didanosine, and enalapril maleate. Permeability values from our *in vitro* diffusion model were compared with the BCS permeability classification and *in vivo* and *in vitro* gastrointestinal drug permeability. The flux rates of the four drugs were influenced by the length of the experiment. Both class 1 drugs showed a significantly higher mean flux rate between 2 and 6 h across the jejunal mucosa compared to the class 3 drugs. The results are therefore in line with the drugs' BCS classification. The results of this study show that the permeability values of jejunal mucosa obtained with the flow-through diffusion system are good predictors of the selected BCS class 1 and 3 drugs' permeation, and it concurred with other *in vitro* and *in vivo* studies.

KEY WORDS: absorption; intestine; *in vitro*; jejunum; permeation.

INTRODUCTION

The oral route remains the most preferred route of administration of new drugs, regardless the tremendous research on alternative drug delivery methods in the last few decades (1). More than 60% of marketed drugs are oral products, despite some shortcomings of this route of administration (2). Oral administration is often preferred due to its convenience, high patient compliance, less stringent production conditions, and lower costs. It is also considered safe, efficient, and easily accessible, with minimal discomfort to the patient compared to other routes of administration. Tremendous growth in the field of genomics and combinatorial chemistry, combined with advances in technological innovations, has led to vast numbers of potential drug candidates. The screening of potential compounds for permeability/absorption, solubility, stability, and biological activity is one of the rate-limiting processes in drug discovery. Reliable *in vitro* models can be applied to determine the permeation of test compounds, which will help avoid the wasting of valuable resources for the development of drugs that are destined to fail in preclinical and clinical phases due to insufficient absorption properties. The pharmaceutical industry therefore requires rapid and accurate methods to screen drug leads for intestinal permeability potential in the early stages of drug discovery (1,3).

Different *in vitro* test methods are available to assess the intestinal permeability of potential drug candidates. Each *in vitro* method has its distinct advantages and disadvantages. Examples of *in vitro* models currently available to study intestinal permeability include the use of animal tissue, isolated membrane vesicles, artificial membranes, cell culture models, intestinal segments, and the everted gut technique. The different types of diffusion equipment available to study *in vitro* tissue permeability include the conventional static Franz cells, where an accurate volume of sample must be removed with a simultaneous media replacement, the Ussing chamber, optimized for the use of high-frequency alternating current stimuli, as well as flow-through diffusion cells (4).

Ninety percent of all absorption in the gastrointestinal tract occurs in the small intestinal region. The human small intestine is approximately 2–6 m long and is divided into three areas, namely, the duodenum, jejunum, and ileum (5). In humans, the jejunum is the principal site for absorption of nutrients and drugs. The jejunum has the largest surface for absorption and is the site of the most active carrier-mediated transport (6). It was therefore decided to use jejunal mucosa for evaluating small intestine permeability in the present study.

The biopharmaceutics classification system (BCS) was introduced in the mid-1990s to function as a scientific framework for classifying active pharmaceutical ingredients based upon their aqueous solubility and intestinal permeability. The BCS classifies drugs in to four different classes according to their aqueous solubility and intestinal permeability characteristics (rate-limiting biopharmaceutical factors) (6,7).

The aims of the present study were to investigate the permeability of excised human jejunal mucosa to four

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different oral drugs using a flow-through diffusion system. The four drugs were selected as representative model compounds of drug classes 1 (high solubility, high permeability) and 3 (high solubility, low permeability) according to the BCS. Furthermore, the objects of the study were to determine the suitability of our *in vitro* diffusion model to evaluate and predict *in vivo* gastrointestinal drug permeability and to compare it with the BCS permeability classification.

MATERIALS AND METHODS

Small Intestine Mucosa

Six human jejunal specimens were obtained from excess tissue removed from two female and four male patients, mean age 43±9 SD (range, 33–54) years, following surgical procedures at Tygerberg Hospital, Bellville, South Africa. The tissue samples were taken from trauma patients (e.g., stab wounds, gunshot wounds), where intestine repairs and construction were necessary. No specimens were obtained where there was clinical evidence of any pathology that might have influenced the permeability characteristics of the intestine. It was beyond the scope of the study to look at the effect of pathophysiological conditions and drug treatment on the transport systems, but it will be an important aspect to investigate in follow-up studies, since transporter systems are affected by these variables.

All jejunal specimens were immediately placed in a transport fluid after removal and kept at 4°C. The transport fluid consisted of a stock solution of Eagle's Minimum Essential Medium (MEM) without L-glutamine and sodium bicarbonate (Gibco, Paisley, Scotland), to which the latter as well as an antibiotic (penicillin/streptomycin, 100 IU/ml) and an antimycotic (amphotericin-B, 2.5 μg /ml) were added prior to using it for the transport of tissue specimens. The intestine tissue was transferred to our laboratory within 2 h (8–10). At the laboratory, the serosal, muscularis externa, and excess connective tissue were trimmed away, using a forceps and scalpel. Specimens from each patient were snap-frozen in liquid nitrogen and stored at −85°C. The frozen mucosal specimens were equilibrated in phosphate buffered saline (PBS; pH 7.4) for 30 min at room temperature, to thaw completely prior to the start of each experiment, after which they were carefully cut into 5×5 mm specimens, so as not to damage the epithelial surfaces.

The Ethics Committee of Stellenbosch University and the Tygerberg Academic Hospital approved the study. Project number: 95/019 (resubmitted and approved on 28/7/03).

Drugs

Examples of drugs that are already classified (BCS) as high and low permeability drugs (class 1 and class 3) were supplied by Aspen Pharmacare (South Africa). The drugs selected from BCS class 1 (high solubility, high permeability) were zidovudine and propranolol HCl, where as the drugs selected from class 3 (high solubility, low permeability) were didanosine and enalapril maleate. Class 1 and 3 drugs were selected, since dissolution is rate limiting to absorption in the case of class 2 and 4 drugs. Official apparatus for *in vitro* drug dissolution testing are already available and formal Food and Drug Administration (FDA) guidelines already exist to provide useful recommendations for their correct use.

Permeability Experiments

The jejunal mucosa specimens were mounted in the flow-through diffusion cells (exposed circular areas 0.039 cm^2) and each permeation study was performed on 7 tissue replicates from each patient (8–10). The mucosal tissue was mounted between the donor and acceptor compartments and the epithelial/mucosal side faced towards the donor compartment. Tissue disks were equilibrated for 10 min in phosphate buffered saline (pH 7.4) at 37°C in both the donor and acceptor compartments of the diffusion cells, before each permeability experiment started. This step was followed by the removal of the PBS from the donor compartment and the addition of 0.5 ml of PBS containing either 5 mg/ml zidovudine, propranolol hydrochloride, didanosine, or enalapril maleate. The drug-containing PBS solutions were prewarmed to 37°C.

The donor compartments were covered with adhesive tape to prevent evaporation. PBS at 37°C was pumped through the acceptor chambers at a rate of 1.5 ml/h and collected by means of a fraction collector, at 2-h intervals for the first 6 h and thereafter at 6-h intervals. The total time of the experiment was 24 h. All permeability studies were performed under sink conditions, i.e., at the completion of each run, the concentration of drug in the acceptor chamber never reached 10% of that in the donor compartment.

The zidovudine, propranolol hydrochloride, didanosine, or enalapril maleate in the acceptor chambers was quantified by means of liquid chromatography/mass spectrometry (LC/MS) analysis.

LC/MS Detection of Zidovudine, Propranolol Hydrochloride, Didanosine, and Enalapril Maleate

Permeant-containing effluent samples, collected from the acceptor compartment of the perfusion apparatus over the 2–24 h sampling intervals, were analysed using LC/MS. Determination was conducted under the following LC/MS conditions:

LC: Waters 2695 Separations Module (Alliance)
MS: Waters API Quattro Micro, electrospray positive

Zidovudine

Column: Waters Xbridge C18 3.5um, 2.1×50 mm
Mobile phase: solvent A, 0.01% trifluoro acetic acid; solvent B, acetonitrile
Injection volume, 10 μl; Retention time, 5.03 min; flow rate, 0.35 ml/min

Propranolol Hydrochloride

Column: Waters Atlantis dC18 3um, 2.1×150 mm
Mobile phase: solvent A, 1% formic acid; solvent B, acetonitrile
Injection volume, 4 μl; retention time, 7.55 min; flow rate, 0.2 ml/min

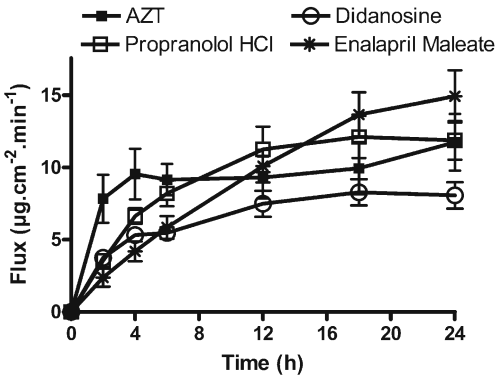

Fig. 1. Overall mean flux values of AZT, propranolol HCl, didanosine, and enalapril maleate across the intestinal mucosa

Didanosine

Column: Waters Xbridge C18, 2.1×50 mm

Mobile phase: solvent A, 0.01% trifluoroacetic acid; solvent B, acetonitrile

Injection volume, 4 µl; retention time, 4.69 min; flow rate, 0.35 ml/min

Enalapril Maleate

Column: Waters Atlantis dC18 3 um, 2.1×150 mm

Mobile phase: solvent A, 1% formic acid; solvent B, acetonitrile

Injection volume, 5 µl; retention time, 8.33 min; flow rate, 0.2 ml/min

Calculation of Flux Values

The flux values (J) across the membranes were calculated by means of the following relationship: $J=Q/(A\times t)$, where Q is the quantity of substance crossing membrane (in µg), A is the membrane area exposed (in cm^2), and t is the time of exposure (in min).

Steady-State Kinetics

Steady state was assumed to have been reached for a particular specimen and chemical compound when no statistically significant differences ($p<0.05$) at the 5% level (t test with Welch's correction) between flux values were obtained over at least two consecutive time intervals.

Statistical Analysis

Nonlinear regression analyses (third-order polynomials) were conducted using a GraphPad Prism, version 4, 2003 computer programme (11). Bonferroni multiple comparison procedures and Bootstrap tests were performed for comparative purposes. A significant level of 5% was used for all tests and comparisons.

RESULTS

The overall mean flux values of zidovudine, propranolol hydrochloride, didanosine, and enalapril maleate across human small intestine mucosa *versus* a 24-h period are shown in Fig. 1. Steady-state fluxes for zidovudine (AZT), propranolol HCl, didanosine, and enalapril maleate were obtained after 3.13, 6.79, 6.52, and 8.35 h, respectively.

The mean flux values for AZT, propranolol HCl, didanosine, and enalapril maleate across the intestinal mucosa are shown as column bars for the time periods 2–4 and 4–6 h in Fig. 2. Both class 1 drugs showed significantly ($p<0.5$) higher mean flux values than the class 2 drugs between 2 and 6 h. Between the time (h) periods 2–4 and 4–6, AZT's mean flux values were respectively 1.8 and 1.7 times higher than didanosine and 2.3 and 1.6 times higher than enalapril maleate. At these same time periods, propranolol HCl's mean flux values were, respectively, 1.3 and 1.5 times higher than didanosine and 1.6 and 1.4 times higher than enalapril maleate. AZT's mean flux values were also on average 1.25 times higher (not significantly, $p>0.5$) than propranolol HCl, and didanosine's mean flux values were on average 1.1 times higher (not significantly, $p>0.5$) than enalapril maleate during the previously mentioned time periods.

The cumulative flux values of the four drugs are shown in Fig. 3, and it is clear that the cumulative flux values of the two class 1 drugs were higher than the two class 3 drugs until the end of the 24-h experiment was reached. AZT and propranolol HCl's (class 1 drugs) cumulative flux values were on average, respectively, 1.7 and 1.3 times higher than didanosine and, respectively, 3.1 and 1.3 times higher than enalapril maleate for the total time of the experiment.

DISCUSSION

Several methods have been proposed and investigated for *in vitro* evaluation and prediction of gastrointestinal permeability of drugs, but no official methods are available at present due to certain limitations (12).

The flux rates of the four drugs were significantly influenced by the length of the experiment. Steady-state fluxes across the intestinal mucosa were obtained for all four permeants during the 24-h period. The steady state fluxes for AZT, propranolol HCl, didanosine, and enalapril maleate were obtained after 3.1, 6.8, 6.5, and 8.4 h, respectively.

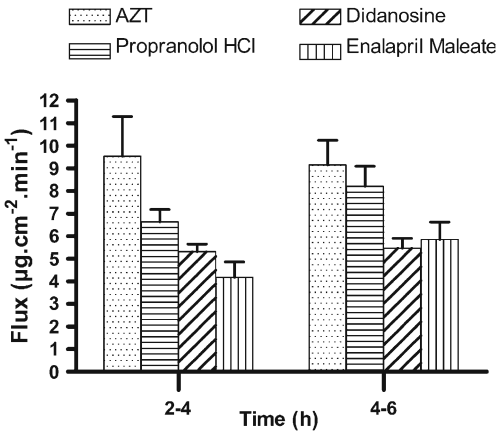

Fig. 2. Mean flux values of AZT, propranolol HCl, didanosine, and enalapril maleate across the intestinal mucosa at different time periods

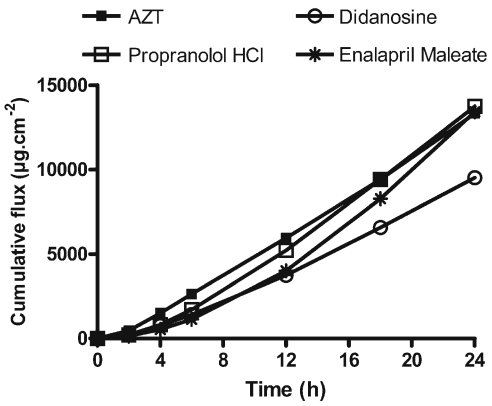

Fig. 3. Cumulative flux values for AZT, propranolol HCl, didanosine, and enalapril maleate across the intestinal mucosa

Even though didanosine has the lowest molecular weight of the four compounds, this compound revealed the lowest overall average flux rate over the entire experiment, which correlates well with its BCS classification as a class 3 drug. Although the oral delivery of didanosine has been extensively studied, the exact reasons for its limited oral bioavailability remain unknown. Didanosine has weak acid stability, and its *in vivo* bioavailability is incomplete and erratic, even when it is co-administered with antacids. The mean fraction available is 43%, with a range of 16–54% (13). Once-daily didanosine doses lead to significant reductions in bioavailability in comparison to the same amount given twice daily, suggesting the involvement of a saturable process (14). The low and variable absorption of didanosine can be partially attributed to the first-pass elimination of didanosine by the liver, presumably by a purine nucleoside phosphorylase enzyme. Degradation of unabsorbed didanosine by the intestinal flora in the lower intestinal tract may also be involved (15). Consistent with this possibility, other authors have shown that the intestinal permeability of didanosine, that is a relatively hydrophilic molecule, is low. The main route for diffusion of didanosine through gastrointestinal epithelium is *via* paracellular transport, and it is regionally dependent, decreasing significantly in the terminal ileum and proximal ascending colon (16,17). Although these studies suggest that slow absorption coupled with degradation in the lower intestine may play a role, the exact reason for the low and variable oral bioavailability of didanosine remains to be conclusively identified.

The mean flux values for AZT, propranolol HCl, didanosine, and enalapril maleate across the intestinal mucosa are shown as column bars for the time periods 2–4 and 4–6 h in Fig. 2. It was decided to choose these specific time periods to compare the mean steady state flux values of the four chosen compounds, since the small intestinal transit time is generally considered to range between 3.5 and 4.5 h (18,19). Both class 1 drugs showed significantly ($p<0.5$) higher mean flux values than the class 2 drugs between 2 and 6 h, but interestingly, no significant differences were found for the rest of the permeability experiment. AZT's mean flux values between the time (h) periods 2–4 and 4–6 were, respectively, 1.8 and 1.7 times higher than didanosine and 2.3 and 1.6 times higher than enalapril maleate. At these same time periods, propranolol HCl's overall mean flux values across small intestine tissue were, respectively, 1.3 and 1.5 times higher than didanosine and 1.6 and 1.4 times higher than enalapril maleate. AZT has a higher molecular weight (267.24 g/mol) than didanosine (236.23 g/mol), but it showed a higher mean flux rate than didanosine for the entire 24-h experiment. These permeability results correlate well with their BCS classification. AZT also reached steady state earlier than didanosine. Huang *et al.* previously reported that AZT permeates the intestine by a Na^+/nucleoside carrier (20). An *in vitro*, brush border membrane vesicle study by Oulianova *et al.* confirmed these results (21). Sinko *et al.* (16,22) ruled out a significant carrier-mediated absorption of didanosine through rat intestinal brush border membrane vesicles, even though transporters are known to play an important role in the disposition of didanosine in other tissues. The carrier system involved with AZT absorption may therefore explain its higher permeability rate across the small intestine.

Both AZT and propranolol HCl belong to BCS class 1, but interestingly, AZT's mean flux values were on average 1.25 times higher than propranolol HCl during the time periods 2–4 and 4–6 h. This may be explained by the carrier-mediated transport of AZT compared to propranolol HCl, which is predominantly absorbed by a passive diffusion mechanism. AZT showed the highest initial flux rate. It therefore seems that the carrier-mediated absorption process of AZT is saturable because AZT reached steady state earlier than the other three drugs, despite its initial highest flux rate. After AZT reached steady state, propranolol HCl's flux rate gradually increased above that of AZT. The results of the present study concur with other *in vitro* and *in vivo* studies that showed that propranolol HCl has a high partition coefficient (23,24). Factors influencing permeability of propranolol HCl *in vitro versus in vivo* will be limited, since propranolol HCl is transported transcellularly, and neither has affinity to cellular efflux or to influx systems (25,26). The high permeability of propranolol HCl is in line with the reported high oral absorption (>90%) (27) and BCS classification as a class 1 drug. The results are in contrast with other authors who found that propranolol exhibited a low *in vitro* permeability. It seems that the flow-through diffusion system used in the present study was able to overcome this problem by having a constant flow of buffer and concentration gradient between the acceptor and donor compartments. It also does not exhibit the problem of an unstirred water layer, which was found in the other studies.

During time periods 2–4 and 4–6 h, the mean flux values of didanosine were very similar to enalapril maleate. Didanosine's mean flux values were on average only 1.1 times that of enalapril maleate, which correlate well with their same BCS classification.

Enalapril maleate is an amino acid ester prodrug, and the transport of enalapril maleate in rats and Caco-2 cells has been shown to be a combination of both passive and active processes, involving a carrier-mediated peptide transport system (28–30). From the results obtained in the present study, it was clear that the order of cumulative flux rate of the four drugs studied through intestinal mucosa were AZT > propranolol HCl > didanosine > enalapril maleate up to the end of the 24 h period (Fig. 3). Enalapril maleate reached steady state last, but at the end of the 24-h period, enalapril

maleate exhibited the highest mean flux rate across intestinal mucosa even though it has the highest molecular weight (492.52 g/mol) of the four test drugs. Despite enalapril maleate's high-end mean flux rate, its cumulative flux rate over the 24-h period of the experiment remained the lowest. The high, mean end flux of enalapril may be explained by the combination of passive and active mucosal transport processes.

Caco-2 cells are the most advanced and frequently used cell model to predict small-intestine drug permeability and absorption (31–33). Compared to Caco-2 cells, the present *in vitro* assay do not have drawbacks such as long cell growth cycles, possibility of microbial contamination, high costs, and relatively wide inter-experiment and inter-laboratory variations, which limit Caco-2 cells as high-throughput screening systems. The present model also offers the possibility to study regional differences in intestinal absorption and the effect of specific transport inhibitors.

The mean flux values of the two class 1 drugs (AZT and propranolol HCl) over the 2–4 and 4–6 h periods of the experiment and the overall cumulative flux results were considerably higher than the respective permeability results from the two class 3 drugs (didanosine and enalapril maleate). By comparing the permeability results of this study with the BCS classification, it seems that the present *in vitro* flow-through diffusion system may have future possibilities for the permeability prediction of class 1 and 3 drugs in humans. The results of this study show that the permeability/flux values obtained with the flow-through diffusion method are good predictors of the small intestine permeability of the four selected drugs. The proposed method allowed obtainment of good results for both high permeability and low permeability compounds and drugs that are absorbed by passive transcellular diffusion, as well as by transport-mediated drug absorption. Previous authors only found a correlation between human *in vivo* and *in vitro* permeability, for drugs absorbed by passive transcellular diffusion. A considerable deviation for transport-mediated drug absorption was found with these studies (34–36).

CONCLUSIONS

The *in vitro* flow-through diffusion model of the present study has proved to be reliable to predict permeability of the four selected drugs and also showed correlation with human *in vivo* data. Such a model may potentially facilitate drug discovery and development, as well as regulatory standards for marketed products. This could reduce costs and time during the development process and reduce unnecessary drug exposure to healthy volunteers. However, an in-depth investigation of pH influence, formulations for the solubilization of the test compounds, and an adequate further enlargement of the set of drugs will be necessary to better evaluate the actual permeability predictive power of the present flow-through diffusion system. Further permeability studies with this model are necessary to enlarge the set of flux values of already BCS-classified drugs, so that clear flux value margins can be established for the specific BCS classes. These established flux value ranges will help to accurately classify the permeabilities of future test compounds. Further studies should also be conducted to compare and validate the present *in vitro* model against Caco-2 cell lines.

The BCS allows a biowaiver for solid, immediate-release (IR) oral formulations with high solubility and high permeability (class 1). These IR compounds are candidates for *in vitro* assessment of bioequivalence as long as they are stable in the gastrointestinal tract and have a wide therapeutic index (6). Instead of conducting expensive and time consuming *in vivo* studies, an *in vitro* test could therefore be adopted as the surrogate basis for the decision as to whether the two pharmaceutical products are equivalent (37). In view of the above considerations and the results found in the present study, it can be concluded that the flow-through diffusion system may have potential usefulness for future *in vitro* bioavailability/bioequivalence (BA/BE) determinations.

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