Human Intestinal Permeability of Piroxicam, Propranolol, Phenylalanine, and PEG 400 Determined by Jejunal Perfusion

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Purpose. To determine the human jejunal permeabilities of compounds utilizing different transport mechanisms using a regional perfusion approach and to establish a standard procedure for determining drug permeability class to be used for the establishment of drug product bioequivalence standards.

Methods. Six healthy male volunteers participated in this study. A multi-lumen perfusion tube was inserted orally and positioned in the proximal region of the jejunum. A solution containing piroxicam, phenylalanine, propranolol, PEG 400 and PEG 4000 was perfused through the intestinal segment at a rate of 3.0 ml/min. Perfusate samples were quantitatively collected every 10 minutes for two 100 minute periods with an intermediate wash out period to determine intra and intersubject variation.

Results. The mean P_{eff} ($\pm$SD) of piroxicam, phenylalanine, propranolol, and PEG 400 were 10.40 $\pm$ 5.93, 6.67 $\pm$ 3.42, 3.59 $\pm$ 1.60, 0.80 $\pm$ 0.46 $\times$ 10^{-4} cm/sec, respectively. The coefficient of variation for the intersubject variability, first and second perfusion periods were: piroxicam, 60.5% and 57.1%; phenylalanine, 52.8% and 57.8%: propranolol, 62.1% and 44.6%; and PEG 400, 81.7% and 42.3%, indicating a slightly lower CV for the second perfusion period in the same subject. The intrasubject CV's between the two perfusion periods were: 19.4%, 21.3%, 23.6% and 41.0% respectively, indicating a smaller intraindividual variation for all compounds studied.

Conclusions. Piroxicam, a nonpolar drug exhibited the highest permeability of the compounds studied. The intrasubject CV was lower than the intersubject CV, indicating consistent permeability estimation within subjects. The methodology is useful for permeability estimation regardless of absorption mechanism and can be used to establish a consistent data base of human permeabilities for estimation of human drug absorption and for establishing the biopharmaceutic permeability class of drugs.

KEY WORDS: intestinal permeability; drug absorption; piroxicam; propranolol; biopharmaceutic classification scheme.

INTRODUCTION

The oral bioavailability of a drug is affected by many factors including: dissolution, transit time, intestinal permeability and first pass metabolism in the gut and/or liver. Intestinal permeability, a key step in drug absorption, quantitates the fundamental transport capacity of the intestinal mucosa for a particular compound. The rate of permeation is dependent upon several factors including the structure and integrity of the intestinal membrane, the physiochemical properties of the drug, and the specific transport mechanisms involved (1–6).

The determination of effective intestinal permeability in humans has recently become more experimentally accessible through the availability of a regional jejunal perfusion system (3–4). This human perfusion system offers several potential advantages, such as minimizing leakage into the test segment, utilizing physiologic flow rates, achieving higher and more consistent recovery and being able to perfuse a segment of known length (10 cm). Furthermore, it has been shown that there is a mass balance in the disappearance and the appearance rate of antipyrine across the human jejunal mucosa and it was also confirmed that sink condition is a valid assumption (3–4).

The main objective of this study was to evaluate the human effective steady state permeability, P_{eff}, of selected compounds utilizing different absorptive pathways using regional jejunal perfusion system. Specifically, we investigated the P_{eff} of the nonpolar drug piroxicam and three marker compounds that undergo different transport processes across the human jejunal mucosa, including paracellular, transcellular passive and carrier mediated transport. Propranolol, which has a high partition coefficient, was chosen as a marker of transcellular passive transport (6). In contrast, PEG 400, a hydrophilic compound, does not partition well into lipid membranes and is therefore suggested mainly to be transported through tight junctions, and hence between the intestinal epithelial cells (7). Phenylalanine, an amino acid, is transported by the amino acid carrier for large neutral amino acids (LNAA) across intestinal epithelium (8). An additional aim of this study was to evaluate the intrasubject and intersubject variability in the determination of permeability for a diverse set of compounds.

MATERIALS AND METHODS

Drugs and Reagents

Piroxicam was obtained from Pfizer, Inc. (New York, NY). Propranolol and phenylalanine were purchased from Ayerst Laboratories, Inc. (Philadelphia, PA) and Twin Laboratories, Inc. (Ronkonkoma, NY), respectively. Polyethylene glycol (PEG) 400, 1000, 1500 and 4000 were obtained from Union Carbide Chemicals and Plastic Company, Inc. (Danbury, CT).

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Mannitol, $NaH_2PO_4 \cdot H_2O$, Na_2HPO_4, glucose, KCl, NaOH and NaCl of USP or NF grade were used to prepare the perfusate and were purchased from Spectrum Chemical Mfg. Corp. (Gardena, CA) and Abbott Laboratories (North Chicago, IL). HPLC grade solvents were used in all of the assays.

Perfusate Composition: The intestinal perfusate contained 0.1 mM piroxicam, 0.003 mM propranolol, 0.06 mM phenylalanine, 12.5 mM PEG 400, 1.5 mM PEG 4000, 35 mM mannitol, 5 mM KCl, 49 mM $NaH_2PO_4 \cdot H_2O$, 21 mM Na_2HPO_4, 10 mM glucose, 45 mM NaCl and 1 mM NaOH. NaOH was used to dissolve piroxicam. The final osmolality of the perfusate was 290 mosm/l and it had a pH of 6.5.

Perfusion Tube

The intestinal perfusions were performed using a multichannel tube designed to study absorption and secretion in the small intestine in humans (Loc-I-Gut™, Synectics, Sweden) as described in more details elsewhere (4). The tube contained six channels and two latex balloons separated by 10 cm making a regional segment for jejunum perfusion.

Subjects

Six healthy male subjects (aged 22–25 years) gave written informed consent to participate in the study. This investigation complied with tenets of the Declaration of Helsinki promulgated in 1964 and was approved by an appropriate Institutional Review Board. The subjects were 22–25 years of age (24 ± 1.6 years) and were within 20% of their ideal body weight (74.8 ± 14.3 kg). Subjects were judged to be healthy based on medical history, physical examination, complete blood count and serum chemistries. Persons with a history of renal, hepatic, gastrointestinal, cardiovascular or psychiatric disease were excluded from study entry, as were subjects with a history of clinical illness within 2 weeks of study entry. In addition, all subjects were medication free, including over-the-counter agents, for at least 3 days prior to the study.

Experimental Procedure

Following a ten hour overnight fast, the perfusion tube was introduced orally after the upper throat was locally anesthetized with benzocaine spray (Bentlich L. P., Wankegan, IL). To facilitate intubation and assure proper placement, the procedure was performed under fluoroscopic examination. In addition, a guide wire was used during the insertion of the tube to ease the passage of the tube through the pylorus. After the tube was positioned in the proximal jejunum, the balloons were inflated with 20–34 ml of the air, making a segment of 10 cm between the two balloons in the proximal jejunum. A gastric tube (size 12, Medovations Inc., Milwaukee, WI) was introduced orally and placed into the dependent portion of the stomach and used to aspirate the gastric contents (Fig. 1).

Initially, the jejunal segment was manually filled with 120 ml of isotonic saline (37°C) and then rinsed with isotonic saline for at least 10 minutes at a flow rate of 3 ml/min using syringe pumps (Harvard Syringe Infusion Pump 2620, C. R. Bard Inc., Southnatick, MA). Subsequently, the drug-containing solution was perfused at a rate of 3 ml/min for two 100 minute periods. The output fluid from the jejunal segment was collected on ice every 10 minutes by gravity drainage. All syringes and output

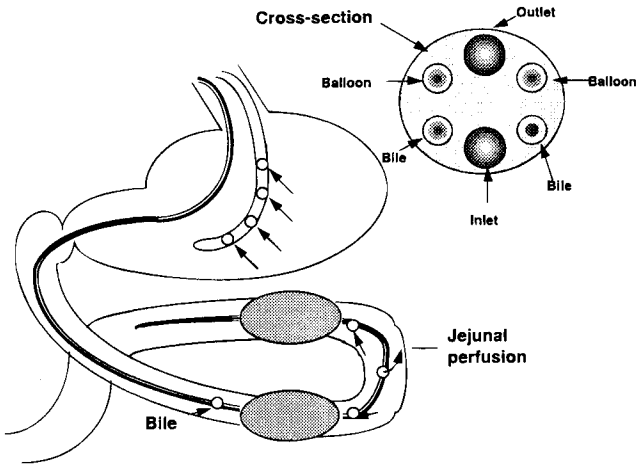


Fig. 1. Regional jejunal perfusion technique.

fluid samples were immediately weighed and stored at −20°C until being assayed. Between the two periods, the intestinal segment was rinsed with 120 ml of isotonic saline. During the study, a vacuum pump was used to facilitate continuous aspiration of gastric contents (from the gastric sump tube) and intestinal fluid (from the proximal port on the perfusion tube). Subjects remained fasted and in a recumbent position until the tubes were removed. Following withdrawal of the tubes, the subjects were fed a standard meal.

Drug Analysis

Concentrations of piroxicam, PEG 4000, propranolol, phenylalanine and PEG 400 were determined by high performance liquid chromatographic (HPLC) methods. The HPLC system consisted of an HPLC pump (Waters 501, Waters Corporation, Milford, MA), an autoinjector (Waters 712, Waters Corporation, Milford, MA), a UV detector (KRATOS Spectroflow 773, Applied Biosystems, Ramsey, NJ), a differential refractometer (Waters 410, Waters Corporation, Milford, MA) and a fluorometer (KRATOS FS970, Applied Biosystems, Ramsey, NJ).

Piroxicam Analysis: The mobile phase was composed of 0.04 M phosphate buffer and methanol (60: 40%, v/v) (9). The separation was performed using an Econosphere C18 column (5 μm, 250 × 4.6 mm, Alltech Associates Inc., Deerfield, IL) with UV detection at 330 nm (10). Samples were prepared by combining 0.1 ml of 1 M hydrochloric acid, 0.1 ml of the internal standard solution (naproxen 1.5 mg/ml) and 8 ml of diethyl ether with 1 ml of the perfusate. After vortexing (1 minute) and centrifuging (3000 rpm for 5 minutes), the ether layer was transferred and then evaporated to dryness under nitrogen. The residue was reconstituted in 2 ml of the mobile phase, vortex-mixed, and 30 μl was immediately injected onto the column. The retention times of piroxicam and naproxen were 5.2 minutes and 7.5 minutes, respectively. The intraday coefficient of variation (CV) was 4.02 and 1.76% at 5 μg/ml and 50 μg/ml, and the interday CV was 8.59 and 9.99%, respectively. The limit of detection of piroxicam was 1 μg/ml.

PEG400, PEG4000, phenylalanine and propranolol were assayed by HPLC using previously reported methods with slight modifications (11–14,18).

Data Analysis

Residence Time Distribution Analysis

To analyze the residence time distribution (RTD), a non-integer mixing tank model was used (17). The RTD analysis provides information about mean residence time and mixing effects. The fractional concentration curves of PEG 4000 (a nonabsorbable marker) in the perfusate leaving the intestinal segment were obtained by fitting the following equation:

$$F = 1 - \left(\frac{r}{r-1}\right)^{n} e^{-((n+r)/rt^*)t} - e^{-((n+r)/t^*)t} \times \sum_{i=1}^{n}\left(1 - \left(\frac{r}{r-1}\right)^{n-i+1}\right)\frac{1}{(i-1)!}\left(\frac{n+r}{t^*}t\right)^{i-1} \quad (1)$$

where F is the fraction of PEG 4000, n is the number of tanks, r is the fraction tank, t is the time and t^* is the mean residence time. The time (t) was calculated by subtracting the residence time in the perfusion tubes, to account for the tube hold up time, and by using the mid-time during the sample collection.

Permeability Calculation

The macroscopic mass balance approach utilizes fundamental mass balance and Fick's first law for estimating permeability and the extent of absorption of drugs (Fig. 2) (15–17). A mixing tank model which represents the completely well stirred flow pattern was applied to calculate the effective intestinal permeability (P_{eff})

$$\frac{dM}{dt} = Q(C_{in} - C_{out}) = A \cdot P_{eff} \cdot C_{out} \quad (2)$$

where C_{in} is the inlet concentration of the drug and C_{out} is the outlet concentration. C_{out} is equal to the concentration in the tube because of the complete mixing. Q is the volume flow rate of the intestine and A is the cylindrical surface area of the intestine. Rearranging Eq. (2) in terms of P_{eff} results in

$$P_{eff} = \frac{Q}{A}\left(\frac{C'_{in}}{C'_{out}} - 1\right) = \frac{Q}{2\pi RL}\left(\frac{C'_{in}}{C'_{out}} - 1\right) \quad (3)$$

where,

$$\frac{C'_{out}}{C'_{in}} = \frac{[\text{Drug}]_{out}}{[\text{Drug}]_{in}} \times \frac{[\text{PEG4000}]_{in}}{[\text{PEG4000}]_{out}} \quad (4)$$

is the fractional concentration of the drug absorbed adjusted for the water transport by the concentration of PEG 4000 (a nonabsorbable marker), L is the length of the intestine and R is the apparent individual radius of the intestine.

The most recent approach for permeability estimation (21), accounts for the individual dynamic segmental volume at each collecting time interval and is estimated from the following equation:

$$V^t_{segment} = \{(\textstyle\sum_0^t \text{PEG4000}_{in} - \sum_0^t \text{PEG4000}_{out})/[\text{PEG4000}_{out}]^t\} + \text{Tube Volume} \quad (5)$$

where Σ_0^t PEG4000$_{in}$ and Σ_0^t PEG4000$_{out}$ are the total masses of PEG4000 entering and leaving the segment respectively from the start of experiment ($t = 0$) to the collecting time interval ($t = t$), $[\text{PEG4000}_{out}]^t$ is the concentration of PEG4000 leaving the segment at time t, the last term represents the tube volume inside the segment. The individual segmental apparent radius is then estimated from the individual segmental volume at each collecting time interval as follows:

$$R^t_{segment} = (V^t_{segment}/L\pi)^{1/2} \quad (6)$$

Steady-State Permeability Estimation

All permeability values after 30 minutes were used for calculations excluding outliers. Z-score test, using 3 standard deviations as a criteria, was applied for outlier detection. This method was then statistically validated in the statistical analysis and RTD analysis results sections.

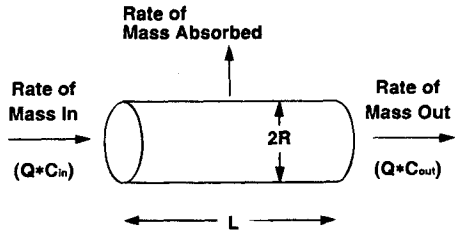


Fig. 2. Macroscopic mass balance in a tube. The rate of mass absorbed equals the difference between the rate of mass flow in and that of mass flow out of the tube.

RESULTS

RTD Analysis

The concentration of PEG 4000 in the outlet perfusate increased rapidly, and reached the steady-state within 30 minutes in all subjects (Fig. 3) validating the steady state starting time. The mean residence times of PEG 4000 in the intestinal segment and the number of tanks of each period for each subject are shown in Table I. Overall, the mean residence time in the human intestinal segment was about 5 minutes. The mean residence time (±SD) of period 2 (3.254 ± 2.361 minutes) was slightly smaller than that of period 1 (5.173 ± 1.899 minutes), however this was not statistically significant. The number of tanks ranged from 1.039 to 7.303 with the mean (±SD) number of tanks being slightly greater for period 1 (4.588 ± 2.085) as compared to period 2 (3.960 ± 2.692).

Estimate of Effective Permeability of Drugs

Fig. 3 shows the mean of the fractional concentrations (C'_{out}/C'_{in}) for the absorbable compounds studied. The estimated values of the effective permeability coefficients of piroxicam, phenylalanine, propranolol and PEG 400 at steady-state ranged from 4.60 to 18.0, 3.41 to 11.5, 2.11 to 6.2 and 0.1 to 2.0 × 10^{-4} cm/sec, respectively. The mean estimates (±SD) of the effective permeability of piroxicam, propranolol, phenylalanine, and PEG 400 were calculated to be 10.40 ± 5.93, 6.67 ± 3.42, 3.59 ± 1.60, 0.80 ± 0.46 × 10^{-4} cm/sec respectively. The average net water flux/cm was 1.74% ± 0.58 and 1.92% ± 0.62 during period 1 and period 2, respectively. Figure-4

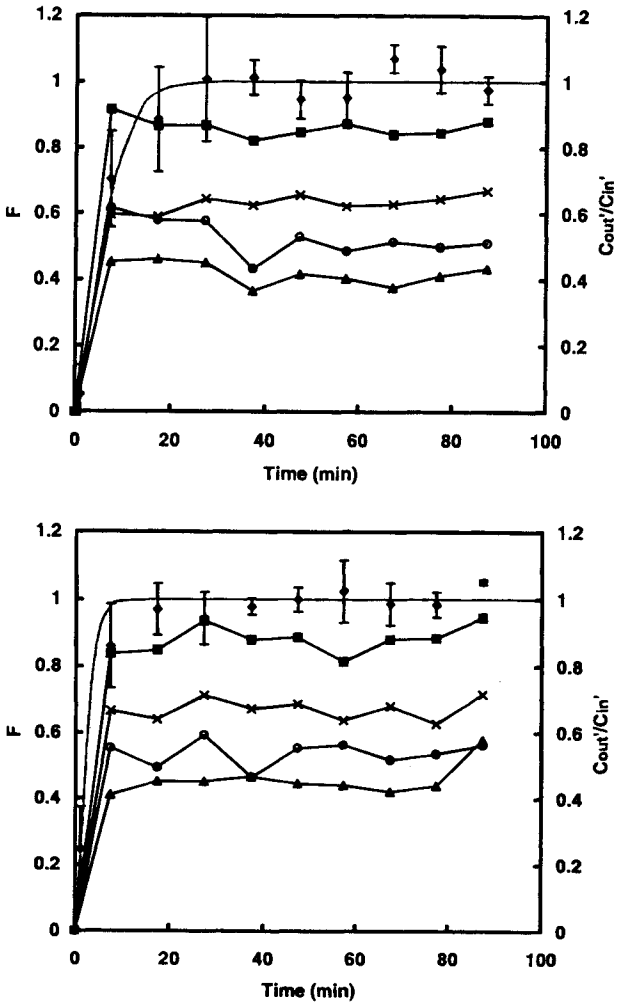
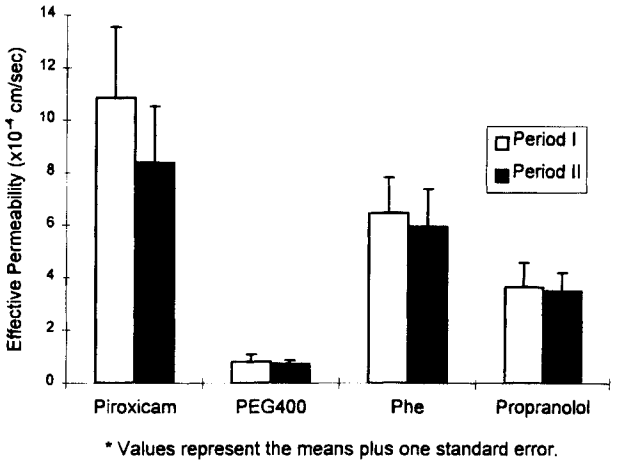


Fig. 3. F-curve of PEG 4000 calculated by residence time distribution analysis (♦, mean ± SD) and mean of fractional concentration of each drug in perfusate (C'_{out}/C'_{in}, ▲: piroxicam, ×: propranolol, ○: phenylalanine, ■: PEG 400).



* Values represent the means plus one standard error.

Fig. 4. Effective intestinal permeability mean values in humans for piroxicam and marker compounds showing no period effect.

shows mean permeability values for piroxicam, PEG 400, phenylalanine (Phe) and propanolol for both periods.

Intraindividual and Interindividual Variation

Mean intersubject coefficient of variation (CV) of P_{eff} within periods 1, and 2 were: piroxicam, 60.5% and 57.1%; phenylalanine, 52.8% and 57.8%: propranolol, 62.1% and 44.6%; and PEG 400, 81.7% and 42.3%, indicating a slightly lower CV for the second perfusion period in the same subject, except for phenylalanine. The mean intrasubject CV values of P_{eff} between the two treatment periods were 19.4%, 21.3%, 23.6% and 41.0% for piroxicam, phenylalanine, propranolol and PEG 400 respectively.

Statistical Analysis

ANOVA with repeated measures design was performed using SYSTAT® to analyze all data simultaneously. The analysis is summarized in Table II. The time effect was not statistically significant validating the steady state assumption. In addition, the period, period-time, and period-subject interaction terms were not statistically significant. Consequently, for the determination of population average permeabilities, a single extended perfusion period is satisfactory.

DISCUSSION

This study further demonstrates the broad usefulness of the regional jejunal perfusion system to determine the intestinal permeability in humans of compounds absorbed by different mechanisms. Specifically, propranolol and phenylalanine compounds which undergo transcellular diffusion and amino-acid carrier mediated transport, respectively had relatively high estimated effective permeabilities. The P_{eff} of phenylalanine was

Table I. Number of Tanks and Mean Residence Time Calculated by Residence Time Distribution Analysis

	Number of tanks		Mean residence time (min)	
	Period 1	Period 2	Period 1	Period 2
Subject A	4.003		5.798	
Subject B	6.232	5.414	5.834	1.595
Subject C	6.267	1.378	5.825	5.281
Subject D	1.248	7.303	6.579	1.381
Subject E	5.191	4.667	1.827	1.681
Subject F		1.039		6.330
Mean ± SD	4.588 ± 2.085	3.960 ± 2.692	5.173 ± 1.899	3.254 ± 2.361

Table II. ANOVA of Piroxicam, Repeated Measures Design Using SYSTAT®

Source of variation	SS	df	MS	F	P
	Between Subjects				
Period	24.3	1	24.3	0.590	0.485
Subject[a]	1698.7	4	424.7	10.323	0.022
Error	164.6	4	41.1		
	Within Subjects				
Time	94.6	6	15.8	0.962	0.471
Time * Period	150.4	6	25.1	1.528	0.212
Time * Subject	426.8	24	17.8	1.084	0.422
Error	393.6	24	16.4		

[a] One subject was not included since the study was stopped after one period only.

assessed at 0.6 mM, a concentration that is about 10 times lower than the Km-value for the LNAA carrier in the rat. Piroxicam is a carboxylic acid with a pKa of 5.1, thus more than 95% ionized at the luminal pH 6.5. The high permeability observed, one of the highest measures to date, is an indication of its apparent high partition coefficient into the membrane even though almost completely ionized.

Previous literature suggests that non-steroidal anti-inflammatory agents may alter the intestinal permeability of selected compounds (19,20) and therefore theoretically, the use of piroxicam may influence the permeability estimates of the different compounds in the present study. For instance, a segmental perfusion system in rats by Krugliak *et al.* in 1990 (19) demonstrated that prostaglandin E2 and non-steroidal anti-inflammatory agents (NSAID, such as acetylsalicylic acid and indomethacin), had opposing effects on the intestinal permeability of PEG 400 and water transport. Although, these authors proposed that these findings were the result of the effect of prostanoids on solvent drag, studies by Choi *et al.* in 1995, suggest that this effect is not universal to all non-steroidal anti-inflammatory agents in humans (20). Specifically, the intestinal permeability of ^{51}Cr-EDTA in healthy volunteers was increased following 7 days of therapy with indomethacin (50 mg three time daily) and sustained release diclofenac sodium (75 mg twice daily). However, the intestinal permeability of ^{51}Cr-EDTA was not significantly influenced by tenoxicam (20 mg daily) or conventional release diclofenac sodium (50 mg three times daily). Although in the present study we can not directly assess whether piroxicam altered the intestinal permeability of the other agents or the water transport, several results indicate that this is not the case. First, piroxicam at the luminal concentration between 0.05–0.1 mM did not have a significant impact on the intestinal mucosa since the net water transport was similar to other studies and similar between phase 1 and 2 perfusion periods. Any short term effect on the mucosa during the course of perfusion would have been observed in the water flux changes during the perfusion and between periods. Second, based on the relatively short duration of our experiment as well as the low concentration of piroxicam employed, one would not expect a significant systemic pharmacodynamic. Finally, if piroxicam had influenced the permeability of the other compounds in the perfusate, we would observe this alteration in differences between period 1 and period 2 permeabilities, while none were observed.

The higher variability for the high permeability piroxicam is due to the lower and more variable exit concentrations. This variability could probably be reduced by increasing the flow rate to reduce the extent of absorption (i.e. result in a higher exit concentration) and to reduce the potential effects of the aqueous boundary layer contribution to the effective permeability. Since the permeability is approaching that of glucose, an aqueous convective-diffusion contribution to P_{eff} is possible (17). However, the flow rate of 3 ml/min represents a compromise flow rate, since increasing the flow rate, will make the determination of the permeability of poorly absorbed compounds difficult (i.e. a small difference between inlet and outlet concentration would be observed for low permeability compounds).

The mean residence time was relatively short i.e. less than 7 minutes in every subject during both perfusion periods and the numbers of tanks relatively small suggesting that the perfusate is reasonably, but not completely, well mixed as soon as it entered the human intestinal segment. These results indicate that the fluid mixing is relatively high in the perfused regional segment of the upper small intestine in humans. Thus a well mixed approximation to the intestinal wall concentration is the most appropriate reference concentration for permeability determination (17). These results also indicate that the time to reach the steady state concentration of PEG 4000, is short i.e. about 3 times the mean residence time (20–30 minutes). The results also, suggests that the mean residence time of period 2 was slightly, although not significantly, smaller than that of period 1. The small differences observed in mean residence time between the two periods may reflect physiological adaptation or relaxation of the intestine with the tube present for a longer period of time. However, the difference in mean residence time between the two treatment periods has a negligible influence on the estimation of the effective permeability coefficient since the P_{eff} at the steady-state was not statistical significantly different between two periods for any of compounds studied.

The two drugs and nutrient coperfused in this study may be considered to all have high permeabilities (22). In fact piroxicam has one of the highest measured permeabilities measured to date. Propranolol, a salt that dissolves rapidly under gastric pH conditions, may be considered a well absorbed drug and hence a high permeability drug class I drug (22,23). In a dosage form that dissolves rapidly under gastric conditions, the absorption rate will be determined by the gastric emptying rate and it's variability in systemic availability will be mainly due to variation in first pass metabolism (22). Thus the systemic availability will not be sensitive to normal formulation differences if rapid dissolution is maintained. Thus, regulatory standards based on *in vitro* dissolution may be sufficient to insure bioequivalence. On the other hand, is piroxicam, an insoluble drug at gastric pH, would be a Class II drug and hence the *in vivo* dissolution rate would determine the *in vivo* absorption rate. An *in vitro in vivo* correlation should be possible, however, this requires development of a dissolution methodology reflective of the *in vivo* controlling process and would probably need to include pH and surfactant media considerations (23,24).

In summary, the closed loop perfusion system can be used to determine the steady state jejunal permeability in humans of multiple drugs. These permeabilities can be used in the establishment of a data base of human permeabilities, determined under standard conditions, for drug absorption estimation

as well as in the determination of biopharmaceutic drug class (BCS) for setting drug regulatory standards. This data base, when fully established, will facilitate both the drug discovery process as well as the setting of drug regulatory standards.

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