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Effect of Water Flux on the Determination of Membrane Permeability Using Single-pass Perfusion

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The single-pass perfusion technique was employed in order to investigate the effect of water flux from the rat jejunum in the normal experimental conditions. Our results suggested that water flux below ± 0.75%/cm of jejunal length was considered normal. Water flux was −0.131 ± 0.311%/cm of jejunal length in a citrate buffer and should be corrected in order to determine the permeabilities of the compounds. Perfusion rate up to 0.5 ml/min had no effects on the permeability of ampicillin. Neither the effective permeabilities nor the wall permeabilities of aminopenicillins were influenced by water flux during experiments in rats.

Keywords—Ampicillin, Amoxicillin, HPLC, Permeability, Water transport

Introduction

Drug absorption studies have utilized animal models and employed *in vivo, in situ,* and *in vitro* techniques. Among them *in situ* single-pass perfusion of intestinal segments in rats is frequently used to study the absorption kinetics[1] and determination of wall permeabilities.[2, 3] There are some advantages on *in situ* perfusion experiments compared to other methods, such as uptake by intestinal rings, uptake by everted gut sacs, and oscillating intestinal perfusion.[4] Gut perfusion bypasses the stomach, thereby eliminating effects of gastric emptying on drug absorption.[5] Using drug solution eliminates dissolution problems during absorption in the gut.[6] Hydrodynamics of the intestine can be carefully defined and kept constant throughout the experiments.[7] Mathematical analysis might be done, since convective diffusion equation governs the single-pass perfusion system.[2, 3, 5, 8-12] The fraction dose absorbed in humans *in vivo* studies can be correlated with rat jejunal drug permeabilities obtains from *in situ* perfusion experiments.[13]

Extensive works were done for effects of luminal stirring,[14] anesthetic regimens,[15] unstirred water layer,[16, 17] perfusion rate,[18, 19] intraluminal radius,[19] longitudinal intraluminal concentration [20] and osmolarity[21, 22] on drug absorption. However there are few reports on the water flux during the *in situ* experiments.[23] Water flux naturally occurs during experiments and the outlet concentrations should be corrected with net water flux in order to determine permeabilities of drugs. The purpose of this study is what is the normal range of water flux during single-pass perfusion. In this report we have also studied the effect of water flux on the determination of membrane permeabilities of two aminopenicillins.

Materials and Methods

Materials

Ampicillin, amoxicillin, and urethane were pu-

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rchased from Sigma Chemical Co. (St. Louis, MO). Polyethyleneglycol (PEG) 3350 was from Fisher Scientific Co. (Fair Lawn, NJ), and ^{14}C-PEG 4000 from New England Nuclear (Boston, MA). Phenol red was purchased from Wako Pure Chem. Ind. (Osaka, Japan). Citric acid and sodium chloride were from Mallincrodt, Inc. (Paris, KY). Monobasic potassium phosphate, acetonitrile, and dibasic sodium phosphate were from J. T. Baker Chemical Co. (Phillipsburg, NJ). All chemicals are analytical or HPLC grades. Ecolite (+), a liquid scintillation cocktail, was from ICN Biomedicals, Inc. (Cleveland, OH).

An appropriate amount of the compound to be studied was dissolved in a citric acid-dibasic sodium phosphate buffer, of which pH was adjusted to 6.5 (a Beckman pH meter, Fullerton, CA) and osmolality to 300 ± 5 mOsm/kg (a Wescor Model 5500 vapor pressure osmometer, Logan, UT). PEG 3550 (0.01 w/v%) with a trace amount of ^{14}C-PEG 4000 was added to the perfusion solution.

***In Situ* Single-Pass Rat Perfusion Experiments**

Single-pass perfusion was done with the same method reported in elsewhere.[10] Briefly, male Charles River Rats, 250~350 g, were fasted for 15 to 18 hours prior to each experiment. Water was given *ad libitum*. Anesthesia was induced by an intraperitoneal injection of a 50% (w/v) urethane solution (1.6 g/kg). The jejunum was cannulated at 2 to 4 cm below the ligament of Treitz and about 10 cm distal to the first incision. The jejunal segment was perfused using a constant infusion pump (Harvard Apparatus, Model 931, South Natick, Mass.) for 2 hours. The perfusate was maintained at 37 ± 1℃ by a water bath (Tek-Pro, American Dade, Miami, FL). During the experiment the rat was kept on a Precision slide warmer (GCA Co., Chicago, IL), and the abdomen was covered with a saline-wetted paper towel and a piece of Parafilm (American Can Co., Greenwich, CT). The perfusion flow rate was 0.08 or 1.0 m*l*/min. Steady-state was achieved in approximately 30 minutes, after which six samples were taken at 10 or 15 minute intervals. After the last sample was taken, the length of the intestine was measured by placing a piece of string along the intestine and measuring the string with a ruler. The data were reported by means (± s.e.m.) from 3 to 5 rats with 2 to 5 determinations per rat.

Analytical Methods

For the water flux measurement, 0.5 m*l* of the sample was mixed with 15 m*l* of scintillation cocktail. The samples were counted by a Beckman LS-9000 counter (Beckman Instruments Inc., Fullerton, CA). In order to determine the effect of perfusion rate on the water flux, phenol red was used instead of PEG 4000. Phenol red was determined using a spectrophotometer. The samples were analyzed by high performance liquid chromatography (HPLC). The HPLC instrumentation consisted of a pump (Spectroflow 400, Kratos Analytical Instruments, Ramsey, NJ), an automatic injector (Waters 712 WISP, Millipore Co., Milford, Mass.), a reverse phase column (Partisil 10-ODS, 25 cm, Whatman Inc., Clifton, NJ), a UV detector (Spectroflow 783 or 773, Kratos Analytic Instruments, Ramsey, NJ), and an integrator (Model 3390A, Hewlett-Packard Co., Avondale, Penn.). The mobile phase consisted of acetonitrile and 0.01 M monobasic potassium phosphate (pH 6.1) in the ratio of 10 : 90 and 5 : 95 for ampicillin and amoxicillin respectively. The flow rate was 1.2 m*l*/min, and the UV wavelength was used 215 nm for ampicillin and 225 nm for amoxicillin respectively. The concentration was determined by a peak height.

Data Analysis

Water Transport—Assuming that ^{14}C-PEG 4000 will not be absorbed from the intestine, the percentage of water transport per centimeter length perfused for each sample was calculated from :

$$\% \text{ Water transport} = \left(\frac{A_{out} - A_{in}}{A_{in}}\right) \cdot \frac{100}{L} \qquad (1)$$

where A_{in} and A_{out} are the disintegrations per minute (dpm) of the inlet and outlet samples, respectively, and L is the length of the jejunum experimented. When phenol red was used as an unabsorbed marker, A_{in} and A_{out} are its concentrations of the inlet and outlet samples, respectively. Positive % water flux means water absorption from the intestine and negative value means water secretion into the intestine.

Estimation of Membrane Permeabilities—The intrinsic membrane absorption parameters were estimated using a modified boundary model approach developed by Johnson and Amidon.[24] Assuming that the difference between the rate of mass flowing into and out of the intestine is equal to the rate of mass absorbed, the dimensionless effective wall permeability, P_{eff}^{*}, is calculated from the steady-state perfusion results :

$$P_{eff}^{*}=\frac{\left(1-\frac{C_{in}}{C_{out}}\right)\cdot Q}{2\pi DL} \quad (2)$$

where C_{in} and C_{out} are the inlet and outlet perfusate concentrations, respectively, D is the diffusion coefficient, L is the length of the intestine perfused, and Q is the fluid flow rate. The perfusate outlet concentration was corrected using Eq. (1). The corrected concentration ratio was kept between 0.85 and 0.95 to estimate the permeability parameters . The dimensionless aqueous permeability, P_{aq}^{*}, is estimated from the film model approximation to the boundary layer results[24] and the dimensionless wall permeability, P_{w}^{*}, is calculated by :

$$\frac{1}{P_{w}^{*}}=\frac{1}{P_{eff}^{*}}-\frac{1}{P_{ag}^{*}} \quad (3)$$

In general, the multiplication of the parameter by R/D results in the dimensionless parameter where R is the radius of the intestine and D is the diffusion coefficient of the compound.

Results and Discussion

Water absorption or secretion during perfusion experiments may change the outlet concentrations of the compound studied. Therefore net water flux needs to be determined in order to estimate wall permeabilities, as seen in Eq (2). There were few reports on the normal range of water flux during experiments. It was shown in the literature that water flux below 0.5%/cm of intestinal length was considered normal.[10] In order to investigate what is the normal range of water flux in the rat jejunum, single-pass perfusion experiments were performed with 282 rats using a citrate buffer kept pH 6.5. Osmolality was kept constant. Fig. 1 shows the distribution of water flux in a histogram. Mean (± s.d.) was −0.131 (± 0.311)%/cm of intestinal length, suggesting water secretion without statistical significance (Table I). Similar result was reported that water efflux was larger than water influx in ch-

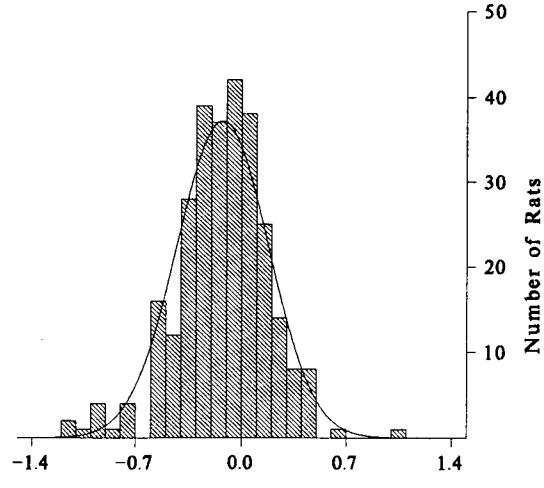

Figure 1—Histogram of water flux in rat jejunum using a citrate buffer during single-pass perfusion experiments. Mean (± s.d.) was −0.131 (± 0.311)%/cm of intestinal length and 95% confidence limits were −0.753 and 0.49 1%/cm of intestinal length. The line represents a normal distribution.

Table I—*Summary of Statistics on Water Flux in the Citrate Buffer during Single pass Perfusion at the Rat Jejunum*

Statistics	Value
Number of rats	282
Minimum	−1.448
Maximum	1.094
Mean	−0.131
Standard deviation	0.311
Standard error	0.019
Skewness	−0.547
Kurtosis	2.309
Coeffcient of variance	−2.384

ronic jejunal loop rats.[23] The 95% confidence limits were −0.753 and 0.491%/cm of intestinal length. The normal water flux of ±0.5% in the literature[10] can be acceptable but more reliable range may be ±0.75%/cm of the intestine. Experimental results with higher water flux than ±0.75%/cm cannot be used for the determination of the permeabilities in the rat. Some rats with higher water flux may be suspected the diarrhea[23] The normal values of water flux may not be the same in different buffer perfusates even though the pH of perfusates is the same. In fact Lu *et al.*[23] have reported that water fluxes in MES buffer and McIlvaine buffer were significantly different, when both *in situ* and chronic isolated loop techniques were employed.

The effect of perfusion rate on the water flux was shown in Fig. 2. Various perfusion rates ranging from 0.08 ml/min to 1.0 ml/min were used. There was no significant water absorption or water secretion at various perfusion rates except at 1.0 ml/min. We don't know the reason why at 1.0 ml/min there appears to be a significant water absorption at this stage. It has been suggested that distention of the intestinal lumen occurs at elevated flow rates. The perfusion rate below 0.5 ml/min seems to be an adequate rates for single-pass perfusion. The optimal rates could be higher perfusion rates in terms of low variabilities.[25] However the perfusion rate should be reduced below 0.5 ml/min in order to maintain a certain range of C_{in}/C_{out} ratios.[24]

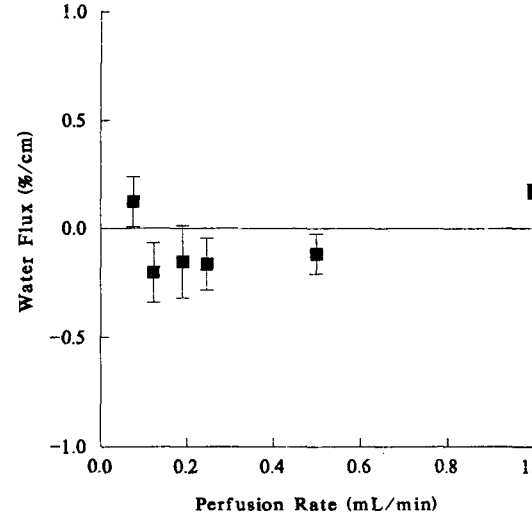

Figure 2—Effect of perfusion rate on the water flux in rat jejunum. Each point represents mean (± s.e.m.) determined from 3 to 8 rats.

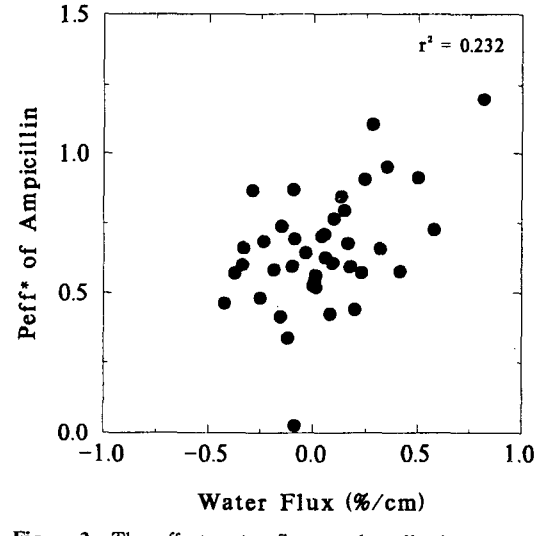

Figure 3—The effect water flux on the effective permeability (P_{eff}) of ampicillin in rat jejunum. There was no correlation between water flux and effective permeability (r^2=0.232).

The effect of water flux on the effective permeability (P_{eff}) of ampicillin in rat jejunum was shown in Fig. 3. There was no correlation between

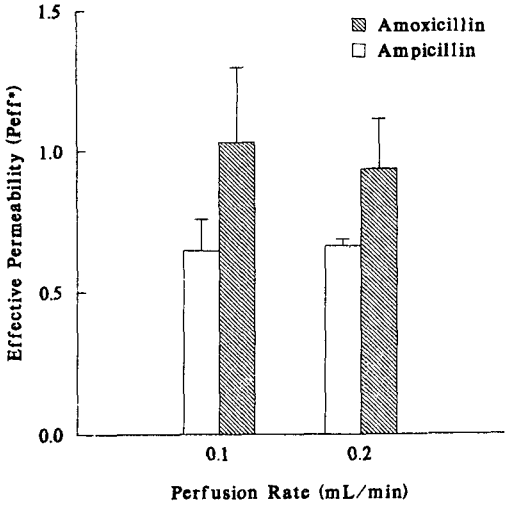

Figure 4—Effect of perfusion rate on the effective permeabilities (P_{eff}^*) of ampicillin and amoxicillin in rat jejunum.

Table II—*Effective (P_{eff}^*), Aqueous (P_{aq}^*), and Intrinsic Wall (P_w^*) Permeabilities of Ampicillin Obtained by in situ Single-pass Perfusion Experiments in Rats*

Rat	P_{eff}^*	P_{aq}^*	P_w^*
1	0.70	2.66	0.55
2	0.62	2.43	0.49
3	0.63	2.67	0.51
4	0.71	2.35	0.55

water flux and effective permeability of ampicillin ($r^2=0.232$). Therefore it can be concluded that permeability was not influenced by normal water flux in rats. Fig. 4 shows the effect of perfusion rate on the effective permeabilities of ampicillin and amoxicillin in rat jejunum. Again a two-fold increase in the perfusion rate had no effect on the effective permeabilities of the compounds, suggesting permeability as an intrinsic parameter of drug absorption. Furthermore perfusion conditions employed in this report had no significant influence on wall permeability (P_w^*) as well as effective permeability of ampicillin (Table II). Since ampicillin is known to be absorbed by a carrier-mediated mechanism,[10] wall permeability is a characteristic of oral absorption that is not changed by water flux in rat jejunum.

In summary, water flux occurs normally during perfusion experiments and should be corrected in order to determine the permeabilities of the compounds. Water flux in normal ranges, ± 0.75 %/cm of the intestine, had no influence either on the effective permeabilities nor on the wall permeabilities of drugs. Perfusion rate up to 0.5 ml/min had no effects on permeabilities.

Acknowledgments

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