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Implications of Density Correction in Gravimetric Method for Water Flux Determination Using Rat Single-Pass Intestinal Perfusion Technique: A Technical Note

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INTRODUCTION

The oral route remains the preferred one for drug administration. Absorption of an orally administered drug is largely dependent on its solubility and permeability through intestinal mucosa.[1,2] Because of regional variability in drug permeation, studies using different intestinal segments have gained importance, as they help in rational "mechanistic" design of drug delivery systems. Various methods like everted sac, intestinal rings, Ussing chamber, single-pass intestinal perfusion technique (SPIP), and Caco-2 cells have been used to study intestinal drug permeability.[3-6] Among these, rat SPIP technique is the most widely used, because of its proximity to in vivo conditions, lower sensitivity to pH variations because of preserved microclimate above epithelial cells, maintenance of intact blood supply to intestine, and good correlation with human absorption data.[7,8] In this technique, the determination of water absorption and secretion, collectively referred to as net water flux (NWF), is important for the calculation of drug permeability.[9-11] Generally, "nonabsorbable" markers like phenol red and radiolabeled (^{14}C) polyethylene glycols are utilized for NWF calculation. However, phenol red may interfere with the transport and/or analytical measurements, and radiolabeled isotopes may raise safety concerns.[12] To overcome these issues, the gravimetric method has been identified as a simpler alternative.

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This method involves determination of the weight of intestinal perfusate collected over a time period and its conversion to a volumetric parameter. After volume of drug solution entering and exiting the intestinal segment is corrected for, the drug's effective permeability coefficient (P_{eff}) is calculated. One approach is to assume the density of both "entering" and "exiting" intestinal perfusate solutions as 1.0 g/mL for calculation of NWF. However, the contribution of cell erosion and mucus can significantly alter the density of perfusate, and an assumption of density value of 1.0 g/mL is expected to introduce errors in NWF calculations. Such errors subsequently can introduce variability in the estimation of P_{eff} of various drug molecules. This problem is expected to be greater in the case of poorly permeable drugs, which inherently show variable permeability.

The present study was aimed at comparing the 2 alternate gravimetric methods—that is, using intestinal perfusate density as 1.0 g/mL (method 1) and using the actual density (method 2) for calculation of NWF in an SPIP study. The impact of these NWF values on the calculation of P_{eff} of 2 poorly permeable drugs, atenolol and furosemide,[13] was also assessed.

MATERIALS AND METHODS

Chemicals

All chemicals used for the study were of analytical grade. Atenolol and furosemide were obtained as gift samples from Panacea Biotech Ltd (Lalru, Punjab, India).

Perfusion Solution

The composition of perfusion solution was similar to that already reported,[11] with concentrations of phenol red,[8] atenolol, and furosemide[11] being 50 mg/L,

0.83mM, and 0.20mM, respectively. The achievement of steady state was determined using phenol red, as reported earlier.[8] The pH of perfusion solution was kept at 6.5 ± 0.02 and osmolality at 290 ± 10 mOsmol/kg.

Rat Single-pass Intestinal Perfusion Technique

Permission for the present study was obtained from the Institutional Animal Ethics Committee (approval number IAEC-01-079). Male Sprague-Dawley rats weighing 220 to 280 g were fasted for 12 to 15 hours prior to the start of the experiment. Water was allowed ad libitum. Anesthesia was induced and maintained for the duration of the experiment by intraperitoneal injection of urethane at a dose of 1.5 g/kg. A midline incision was made on the abdomen and an approximately 15- to 20-cm length of jejunum was selected and cannulated on both sides. The animal was maintained at 37°C throughout the experiment by focusing a table lamp as a source of heat. The exposed segment was covered with a cotton pad soaked in normal saline and then with aluminum foil to prevent evaporation of fluids. Initially, the intestinal segment was washed with isotonic saline (37°C) until the outlet solution was clear. A bolus dose of 3 to 5 mL of drug solution, for equilibration of intestinal segment, was then given, and thereafter the drug solution was perfused at a constant flow rate of 0.2 mL/min using a peristaltic pump (P-1, Pharmacia Biotech, Kwai Chung NT, Hong Kong). The intestinal perfusate samples were collected at 15-minute intervals for a duration of 90 minutes in preweighed 5-mL glass vials. The length of intestinal segment studied was measured at the end of the experiment, and its radius was taken to be 0.18 cm.[14] Finally, the animals were killed by excess of ether. The study was performed in 6 animals for both the drugs, and results are presented as average values.

Analytical Methods

All the samples were analyzed for phenol red and drug concentrations using validated high-performance liquid chromatography (HPLC) analysis. The HPLC system (Shimadzu Corporation, Kyoto, Japan) consisted of a system controller (SCL 10A), pump (LC-10AT), a degasser (DGU 14A), an autosampler (SIL-10AD), a column oven (CTO 10AS) and a UV detector (SPD-10AP) with Class-VP (Release 6.10) software. The analytical column used was Lichrospher 100 RP-18e, 250 × 4, 5 μm (Merck, Darmstadt, Germany). Phenol red was detected at 434 nm, using acetonitrile:20mM pH 6.5 phosphate buffer (20:80) as mobile phase, at a flow rate of 0.8 mL/min. Both atenolol and furosemide were detected at 276 nm, using acetonitrile:50mM pH 6.5 phosphate buffer (20:80) as mobile phase, at a flow rate of 0.7 mL/min.

The HPLC methods for drug analysis were validated for various parameters. For atenolol, linearity was established in the range of 60 to 300 μg/mL (r^2 = 0.9984), with 101.54% accuracy, and 0.12% relative standard deviation (RSD) for precision in terms of repeatability. For furosemide, linearity was established in the range of 20 to 100 μg/mL (r^2 = 0.9991), with 97.92% accuracy, and 0.30% RSD for precision in terms of repeatability.

NWF Determination

The absorption/secretion of water during the experiment was studied by correcting for density changes. The density of collected samples was determined by weighing the contents (using an electronic weighing balance [AG285, Mettler Toledo, Greifensee, Switzerland]) of a known volume of perfusate (using a precalibrated micropipette [Finnpipette, Labsystems, Helsinki, Finland]).

Calculation of NWF was done from intestinal perfusate samples collected over 30 to 90 minutes, the time required for achievement of steady state in concentrations of phenol red. NWF values were determined by 2 methods, as described below.

Method 1: Gravimetric Method

$$NWF = [1 - (Q_{out}/Q_{in})] \cdot Q_{in}/l \quad (1)$$

where Q_{out} and Q_{in} are the measured flows (mL/min) of exiting and entering intestinal perfusates, respectively, for the specified time interval using intestinal perfusate density as 1.0 g/mL; and l is the length (cm) of intestinal segment studied.

Method 2: "Density-corrected" Gravimetric Method

$$NWF' = [1 - (Q'_{out}/Q_{in})] \cdot Q_{in}/l \quad (2)$$

where Q'_{out} is the measured flow (mL/min) of exiting intestinal perfusate for the specified time interval using the actual intestinal perfusate density (g/mL).

Apart from this traditional method, calculations were also attempted using a multiple linear regression

(MLR) method[15] (Jandel SigmaStat statistical software, version 2.0, Jandel Scientific Software Corp, San Rafael, CA), which provided an objective test for achievement of steady state; also, the method's error estimate included multiple components of variability. The model below was fit to data using the MLR method:

$$NWF_{ri} = \alpha_r + \beta t_i + \varepsilon_{ri} \quad (3)$$

where α_r is the intercept for the *r*th rat, β is the slope, t_i is the *r*th time point, and NWF_{ri} and ε_{ri} are the NWF and residual, respectively, for the *r*th rat at the *i*th time point. The variance of average value of NWF (Var NWF_{avg}) was calculated using Equation 4:

$$\text{Var } NWF_{avg} = (\sigma^2 + m\sigma_r^2)/(m.n) \quad (4)$$

where σ^2 and σ_r^2 are the within-rat and rat-to-rat variance components, respectively; m is the number of time points; and n is the number of rats used in the perfusion.

Drug Permeability Determination

Calculation of P_{eff} was also done from intestinal perfusate samples collected over 30 to 90 minutes. The P_{eff} of drugs was calculated using Equation 5:

$$P_{eff} = [-Q_{in} \,.\, \ln (C_{out(corr)}/C_{in})]/A \quad (5)$$

where Q_{in} is the flow rate (mL/min) of inlet solution, C_{in} is the concentration (µg/mL) of drug in the entering solution, $C_{out(corr)}$ is the concentration (µg/mL) of drug in the exiting solution corrected for water flux, and A is the surface area (cm^2) of the intestinal segment studied.

The $C_{out(corr)}$ was calculated by 2 methods:

Method 1: Gravimetric Method

$$C_{out(corr)} = C_{out} \,.\, Q_{out}/Q_{in} \quad (6)$$

Method 2: "Density-corrected" Gravimetric Method

$$C'_{out(corr)} = C_{out} \,.\, Q'_{out}/Q_{in} \quad (7)$$

Statistical Analysis

TP_{eff} values are represented as mean ± standard deviation for n experiments. T procedure was applied on the obtained data for detecting any outliers, which were not considered for calculations. A paired Student *t* test was applied to assess any statistically significant difference between the NWF and P_{eff} values as determined by methods 1 and 2. Statistical differences were assessed at 95% confidence intervals.

RESULTS AND DISCUSSION

Atenolol and furosemide are poorly permeable drugs. Experimental determination of the intestinal permeability coefficient involves high animal-to-animal variability. P_{eff} determination using the rat SPIP technique is a well-established means of assessing the drug's intestinal permeability in normal physiological conditions, as well as in the presence of permeation enhancers. The present research studied the influence of density of exiting intestinal perfusate on NWF and P_{eff} of drug molecules.

The results shown in **Tables 1, 2,** and **3** for each animal are the average values obtained from samples collected at intervals of 30 to 45, 45 to 60, 60 to 75, and 75 to 90 minutes. The density of exiting intestinal perfusate was found to be 1.0284 ± 0.0016 g/mL during the 30- to 90-minute interval of the experiment. The intestinal NWF values determined by methods 1 and 2, and by traditional and MLR methods, are compared in **Table 1**. Mean intestinal NWF value was found to be higher with method 2 (38.49 ± 17.45 µL/hr/cm) as compared to method 1 (19.46 ± 18.23 µL/hr/cm) (**Figure 1**). These results clearly show a 2-fold difference in mean values for nearly similar standard deviation values, as determined by the 2 methods. This difference between the NWF values determined by the 2 methods was found to be statistically significant ($P < .001$). In addition, method 2 had a smaller coefficient of variation (CV), with values of 93.69 and 45.34 for methods 1 and 2, respectively. Also, the traditional and MLR methods of calculation gave comparable average values and error. The observed differences in the density and water flux values highlight the impact of density correction for exiting intestinal perfusate in the calculations. The influence of NWF was also tested on the intestinal permeability of 2 poorly permeable drugs, atenolol and furosemide. Mean P_{eff} values of atenolol (**Table 2**) and furosemide (**Table 3**) were found to be significantly higher ($P < .001$) and in good agreement with reported values[11] using method 2 as compared to method 1, with lower CV in method 2. Atenolol and furosemide are poorly permeable through intestinal mucosa, and any discrepancy in P_{eff} calculation, brought herein by density of intestinal perfusate, is bound to give variable results.

Table 1. Comparison of Intestinal Water Flux (μL/hr/cm) by 2 Different Methods (n = 6)*

Animal No.	Method 1		Method 2		% Difference
	Traditional	MLR	Traditional	MLR	
1	19.45	19.37	24.93	24.71	50.65
2	48.94	48.95	59.18	59.36	23.98
3	–8.56	–8.59	23.14	23.19	185.77
4	19.81	19.62	40.36	40.19	48.69
5	16.93	16.95	36.24	36.75	61.16
6	20.16	20.32	45.19	45.15	42.33
Average	19.46†	19.44†	38.49†	38.22†	
SD	18.23	18.24	17.45	13.49	
SE‡	7.44	7.45	7.12	5.51	
% CV	93.69	93.84	45.34	35.28	

*MLR indicates multiple linear regression; CV, coefficient of variation; Var NWF_{avg}, variance of average value of NWF.
†Significantly different (Student paired *t* test $P < .001$).
‡For the traditional method, SE = SD divided by the square root of n; for the MLR method, SE = SD divided by the square root of Var NWF_{avg}.

Table 2. Permeability Coefficient ($\times 10^{-4}$ cm/sec) Values for Atenolol (n = 6)*

Animal No.	Method 1	Method 2	% Difference
1	0.008	0.082	90.24
2	0.048	0.092	47.83
3	0.041	0.089	53.93
4	0.004	0.042	90.48
5	0.023	0.064	64.06
6	0.026	0.086	69.77
Average	0.025†	0.076†	
SD	0.017	0.019	
% CV	69.74	25.44	

*CV indicates coefficient of variation.
†Significantly different (Student paired *t* test $P < .001$).

CONCLUSION

The rat SPIP technique is a widely accepted method for the determination of intestinal drug permeability. The calculation of P_{eff} using this technique involves correction for water flux across the intestinal membrane during the experiment. Gravimetric water flux calculations are often based on the assumption of a perfusate density of 1.0 g/mL. The possibility of abrasion of the intestinal mucosal surface caused by cannulation and flow of perfusion solution may lead to entry of certain soluble and insoluble components in the exiting perfusate, which may alter its density. Assumption of intestinal perfusate density as 1.0 g/mL for calculation of NWF can introduce an error, which can be translated into erroneous P_{eff} values. The gravimetric method employing "density corrections" in estimating NWF for drug intestinal permeability determination is more reliable. We feel that density differences in intestinal perfusate would be quite pertinent for poorly permeable drugs, especially during studies on (1) the effect of permeation enhancers on drug absorption, and (2) drug-drug interaction at the absorption level. These studies may involve a significant effect of experimental parameters on a drug's permeability, which may be underestimated without proper correction for intestinal water flux.

Table 3. Permeability Coefficient ($\times 10^{-4}$ cm/sec) Values for Furosemide (n = 6)*

Animal No.	Method 1	Method 2	% Difference
1	0.040	0.099	59.60
2	0.073	0.120	39.17
3	0.043	0.080	46.25
4	0.002	0.058	96.55
5	0.069	0.137	49.64
6	0.045	0.108	58.33
Average	0.045†	0.100†	
SD	0.025	0.028	
% CV	56.13	28.18	

*CV indicates coefficient of variation.
†Significantly different (Student paired *t* test $P < .001$).

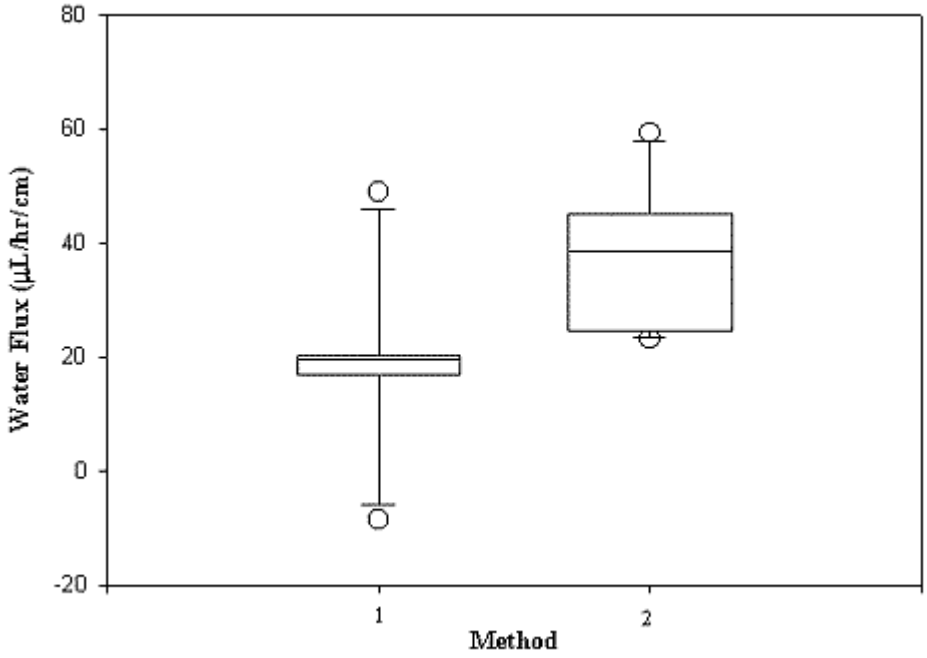

Figure 1. Box plots of NWF calculated by the 2 methods.

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