Comparison of the Gravimetric, Phenol Red, and 14C-PEG-3350 Methods to Determine Water Absorption in the Rat Single-Pass Intestinal Perfusion Model

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ABSTRACT This study was undertaken to determine whether the gravimetric method provided an accurate measure of water flux correction and to compare the gravimetric method with methods that employ nonabsorbed markers (eg, phenol red and ^{14}C-PEG-3350). Phenol red, ^{14}C-PEG-3350, and 4-[2-[[2-(6-amino-3-pyridinyl)-2-hydroxyethyl]amino]ethoxy]-, methyl ester, (*R*)-benzene acetic acid (Compound I) were co-perfused in situ through the jejunum of 9 anesthetized rats (single-pass intestinal perfusion [SPIP]). Water absorption was determined from the phenol red, ^{14}C-PEG-3350, and gravimetric methods. The absorption rate constant (k_a) for Compound I was calculated. Both phenol red and ^{14}C-PEG-3350 were appreciably absorbed, underestimating the extent of water flux in the SPIP model. The average ± SD water flux (μg/h/cm) for the 3 methods were 68.9 ± 28.2 (gravimetric), 26.8 ± 49.2 (phenol red), and 34.9 ± 21.9 (^{14}C-PEG-3350). The (average ± SD) ka for Compound I (uncorrected for water flux) was 0.024 ± 0.005 min^{-1}. For the corrected, gravimetric method, the average ± SD was 0.031 ± 0.001 min^{-1}. The gravimetric method for correcting water flux was as accurate as the 2 "nonabsorbed" marker methods.

KEYWORDS: intestinal perfusion model, permeability, rat, water flux, multiple linear regression.

INTRODUCTION

Perhaps the most-used classic technique employed in the study of intestinal absorption of compounds has been the single-pass intestinal perfusion (SPIP) model[1] . In this model, a solution containing the compound of interest is perfused through a section of the jejunum, with the blood supply left intact. Disappearance of the compound from the perfusate is attributed to intestinal absorption. Because water absorption and secretion during the perfusion may introduce errors in the calculated absorption, various water flux correction methods have been published.

Water flux correction usually involves the co-perfusion of a "nonabsorbed" marker. Whether the marker was a dye (eg, phenol red) or a radioactive isotope (eg, ^{14}C-PEG-3500), the added complexity was often not trivial. For example, phenol red may interfere with the transport or analytical measurement of some compounds (data on file), and radiolabeled isotopes add another level of regulations and safety concerns to the studies.

Because of the aforementioned shortcomings of phenol red (the traditional method at this site) and the interest in switching to a simpler method, the authors determined whether the comparatively simple gravimetric method was equivalent to other methods for water flux correction.

MATERIALS AND METHODS

Materials

(4-{2-[2-(6-Amino-pyridin-3-yl)-2-hydroxy-ethylamino]-ethoxy}-phenyl)-acetic acid methyl ester (**Figure 1**) (Compound I, Pfizer Central Research, Groton, CT) has a molecular weight of 345 d and an aqueous solubility of 150 μg/mL at pH 6.5. Phenol red (lot #100H3667, Sigma Chemical Co, St Louis, MO), PEG-3350 (Carbowax Sentry Polyethylene Glycol, lot #15599938, 3350 Flake NF, FCC Grade, Dow Chemical), and radiolabeled PEG-3350 (NEC-473 Polyethylene Glycol [1,2_^{14}C]-, lot #2967-242, New England Nuclear) were used as is. The pH of a 0.9% sodium chloride injection (USP) (lot #C214452, Abbott Laboratories, Abbott Park, IL) was adjusted to 6.5 using NaOH. All other supplies were of an appropriate grade for their use.

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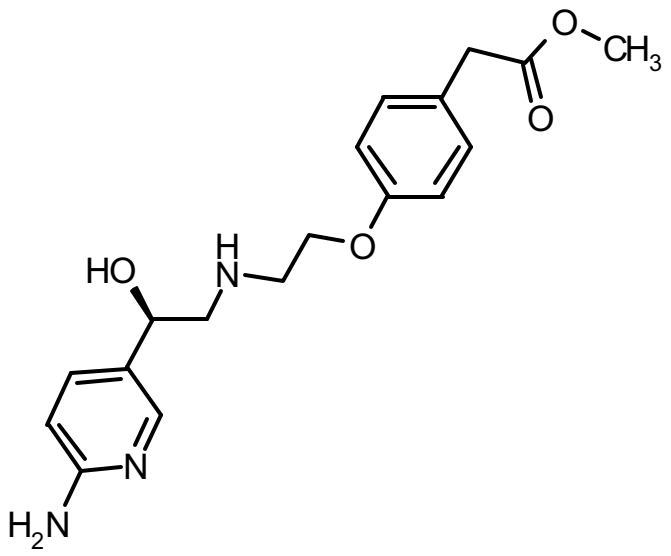

Figure 1. Structure of Compound I.

Assays

^{14}C-PEG-3500. A 100 µL aliquot of samples containing ^{14}C-PEG-3350 was mixed with 15 mL scintillation cocktail (Ready SafeT, BeckmanCoulter, Inc., Fullerton, CA) was detected by liquid scintillation counting (LKB 1217 RackBeta, 10 minutes) and disintegrations per minute (DPM) quantitated by external standardization.

Phenol red and Compound I. A 1.0 mL aliquot from each perfusate sample containing Compound I was diluted with 2.0 mL mobile phase (0.01 M KH_2PO_4 , 0.01 M Na_2PO_4 , 0.8 mL/liter TEA: ACN) before being injected (100 µL, Iso-2000 Perkin Elmer, Bodenseewerk Perkin Elmer GmbH, Shelton, CT, Bundesrepublik Deutschland) and pumped (1 mL/min, LDC Analytical ConstaMetric 3200 Solvent Delivery System, (Spectra-Physics, LDC, Mountain View, CA) through a Dupont (Chadds Ford, PA) Zorbax RX-C18 4.6 mm x 15 cm (part #883967) column. Compound I was detected at 222 nm (LDC Analytical Spectra-Monitor 3200 (Spectra-Physics, LDC, Mountain View, CA) and estimated by peak height (Perkin Elmer Nelson, Model 1020). The retention times of phenol red and Compound I were 2 and 4 minutes, respectively.

Perfusion

The apparatus, which was constructed in-house, consisted of a jacketed aluminum housing and cannula maintained at 37ºC. The perfusate, containing 50 µg/mL of Compound I, 0.1% (wt/vol) unlabeled PEG-3350, 0.02 µCi/mL ^{14}C-PEG-3350, and 0.2 mg/mL phenol red, was pumped (Harvard Smart pump, Harvard Apparatus Inc, South Natick, MA) from a high-density polypropylene 60-mL syringe (Becton Dickinson, Franklin Lakes, NJ) through the apparatus at a nominal 0.2 mL/min for 15 minutes before connecting to the intestine. It had been previously determined that in the rare occasion where nonspecific binding of compound to apparatus had occurred, it was essentially saturated during this perfusion. A sample from the stock drug solution ("stock") and from the apparatus (at the end of the 15-minute initial equilibration period, "pre") was collected and assayed.

The research adhered to the "Guide for the care and use of laboratory animals"[2] and was therefore in accordance with institutional guidelines. Male Sprague-Dawley rats (4-9 per compound) weighing approximately 290-330 g were fasted 12-17 hours (water ad libitum) prior to each perfusion study. The surgery for the SPIP of the rat jejunum was performed as described in detail elsewhere[3]. Briefly, deep anesthesia was induced and maintained for the duration of the procedure with intramuscular urethane (50% vol/vol, 5.0 mL/kg, Sigma Chemical Co, lot #20H0481). A 10-cm section of the proximal rat jejunum was located, gently flushed with saline, and attached to the perfusion assembly. The animals were kept warm with heating pads, and placing parafilm on the abdomen minimized dehydration.

The perfusate samples were collected in 1-dram, tared, glass vials and then weighed. Samples of the exiting perfusate were taken every 15 minutes, for up to 90 minutes. After the 90-minute sample was collected, the animals were euthanatized with a cardiac injection of saturated potassium chloride, the intestine was removed, and a second 15-minute control sample was collected through the perfusion apparatus (designated "post"). ^{14}C-PEG-3350, phenol red, and drug concentrations were immediately determined in samples as described above.

Calculation of k_a

The k_a was calculated using the following equation [4]:

$$k_a = (1 - \frac{C_{out}}{C_{in}}) \bullet \frac{Q}{V} \quad (1)$$

where C_{out} was the concentration of the compound measured in the exiting perfusate at the specified time interval (30, 45, 60, 75, or 90 minutes); C_{in} was the average concentration of the drug measured in the pre, post, and stock samples; Q was the measured perfusion rate (~0.2 mL/min); and V was the volume of the perfused segment (1.26 mL)[4]).

The model below was then fit to the k_a data using the method of multiple linear regression.

$$k_{ari} = \alpha_r + \beta\, t_i + \varepsilon_{ri} \quad (2)$$

where a_r is the intercept for the r^{th} rat (r = 1, 2, 3, ...n), ß is the slope for the compound, t_i is the i^{th} time point (i = 1, ... , 5), kari is the rate constant for the r^{th} rat at the i^{th} time point, and e_{ri} is the residual for the r^{th} rat at the i^{th} time point. The e_{ri} were assumed to be independent and distributed as normal random variates with xmean zero and variance $?^2$. This model fit a straight line to the results of each animal, forcing the slopes for a compound to be parallel. The slope in this model was used to reduce systematic experimental bias that was a function of time.

The studentized residuals (the residual divided by the model standard error) were evaluated to determine whether any statistically significant outliers were present in the individual rat data sets. An outlier was judged as significant if its value was greater than a critical value of the *t* distribution and was removed from further analysis. One is always reluctant to ignore outliers; therefore, outliers should be examined for apparent cause. Once the outliers were removed, the model was used to determine whether k_a was dependent on time (ie, whether steady state was achieved). If k_a had a significant dependence on time, the data associated with the earliest time segment were removed for all rats and the analysis repeated. The steady state condition was considered satisfied only if 3 or more time segments remained and k_a was found not to be dependent on time.

All the remaining k_a data were then used to estimate the variance of the average k_a based on the random effects model of equation 2. The random effects estimate provided a rat-to-rat variance component and a within-rat variance component. These were used to calculate a variance of the mean value of k_a:

$$Var\bar{k}_a = \frac{m * \sigma_r^2 + \sigma^2}{n * m} \quad (3)$$

where n is the number of animals used per compound, m is the number of time points, σ_r^2 is the inter-rat time variability, and σ^2 is the pooled intra-rat variability. The standard error (SE) of k_a is

$$SE_{ka} = \sqrt{Var\bar{k}_a}\,. \quad (4)$$

Water flux corrections

Water absorption or secretion (flux) was measured by 3 methods: gravimetric, phenol red, and ^{14}C-PEG-3500. In these methods, C_{out} was corrected for water flux (through the use of a factor described below) and was then designated as C_{cor} . The corrected k_a was then calculated using equation 1, where C_{cor} was substituted for C_{out} .

Corrected k_a via Gravimetric Correction Method

C_{cor} was calculated from

$$C_{cor} = C_{out} \bullet \frac{Q_{out}}{Q_{in}} \quad (5)$$

where Q_{in} was the measured flow entering the intestine and Q_{out} was the measured perfusate exit flow (net weight/15 min, assumed density of 1.0 g/mL) for the specified time interval.

Corrected k_a via Phenol Red Correction Method

C_{cor} was calculated from

$$C_{cor} = C_{out} \bullet \frac{CPR_{in}}{CPR_{out}} \quad (6)$$

where CPR_{in} was the average phenol red concentration measured in pre, post, and stock samples and CPRout was the phenol red concentration averaged from the exiting perfusate samples.

Corrected k_a via 14CPEG-3500 Correction Method

C_{cor} was calculated from

$$C_{cor} = C_{out} \bullet \frac{PEG_{in}}{PEG_{out}} \quad (7)$$

where PEG_{in} was the average DPM measured in pre, post, and stock samples and PEG_{out} was the DPM averaged from the exiting perfusate samples.

RESULTS

Comparison of water flux correction method

A detailed comparison of water flux correction methods was possible for Compound I because a large number (n = 9) of SPIP preparations was completed. Only water *absorption* (ie, net *positive* water flux) was observed for Compound I when the gravimetric correction method was used. However, when correcting for water flux using the phenol red or PEG methods, water secretion was apparent in 1 or more preparations (**Table 1**).

The average water flux estimated by the gravimetric method (68.9 ± 28.2 µL/h/cm) was approximately double the flux estimated by either the PEG (34.9 ± 21.9 µL/h/cm) or the phenol red (26.8 ± 49.2 µL/h/cm) methods (**Figure 2**). The difference between the average flux as estimated by the gravimetric method and the average flux estimated by either the PEG or phenol red methods was statistically significant ($p < 0.05$). Water flux determined by the gravimetric method had the smallest coefficient of variation (CV) (%CV = SD/average x 100) at 41%. The CVs were 184% and 63% for the phenol red and PEG methods, respectively. Precautions were taken to minimize systematic errors. Evaporation of fluids or collection of mucous could not explain the greater water flux estimated by the gravimetric method. For example, if evaporation occurred, the perfusate sample would appear to reflect *less* water flux. Conceptually, mucous could contribute to the sample's weight (changing the sample's density), potentially overestimating water flux. However, in a

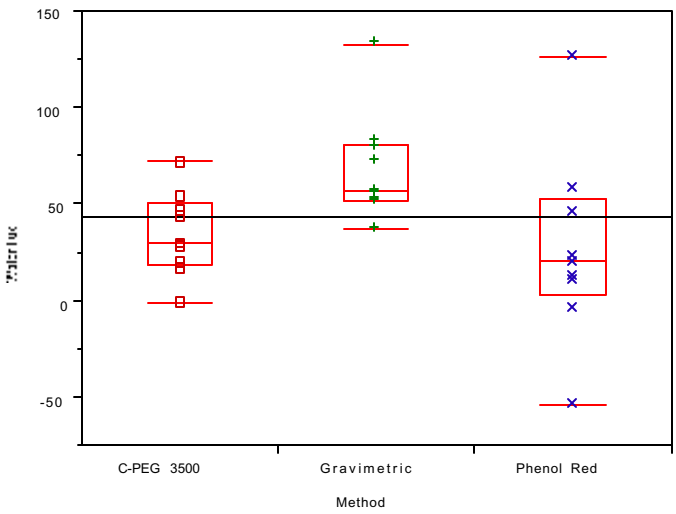

Figure 2. Box-plots of water flux by method.

Table 1. Comparison of Water Flux (mL/h/cm) and ka (min-1) Estimated by the Gravimetric, Phenol Red, and 14C-PEG 3500 Methods for Compound I (n = 9 Animal Preparations)

Preparation #*	Gravimetric		Phenol Red		^{14}C-PEG 3500	
	Flux	k_a	Flux	k_a	Flux	k_a
1	72	0.000	46.56	-0.003	17.52	-0.007
2	79.2	0.012	10.8	0.004	54.36	0.009
3	51.6	0.047	126.72	0.054	28.92	0.045
7	133.2	0.026	23.04	0.013	72.36	0.019
8	52.8	0.013	-4.08	0.006	30.36	0.010
9	82.8	0.026	58.32	0.024	46.68	0.022
10	56.4	-0.002	20.64	-0.007	44.28	-0.003
11	55.2	0.018	12.84	0.013	20.4	0.014
12	37.2	0.032	-54	0.022	-0.72	0.028
Average	68.9	0.019	26.8		34.9	
SD	28.2	0.016	± 49.2	0.018	21.9	0.016
CV(%)	41	80	184	130	63	104

*Data from preparations 4-6 were lost during assay.
CV, coefficient of variation; ka, absorption rate constant

Table 2. Comparison of Water Flux (mL/h/cm) Estimated by the Gravimetric and Phenol Red Methods for 9 Compounds (n = 3 Animal Preparations per Compound)

NCE[a]	Gravimetric	Phenol Red
A	45.9	61.0
B	87.0	107.7
C	90.5	88.8
D	99.5	78.5
E	35.5	-21.7
F	59.2	52.2
G	85.2	133.8
H	106.0	62.1
I	68.9	26.2

[a] New chemical entity.

representative number of experiments, the density of exiting perfusate was measured and found unchanged. Although it is possible that under some circumstances an irritating substance may cause massive mucous shedding, it is the considerable experience of these authors that other indicators (eg, presence of blood in the perfusate, large fluctuation in perfusate flow) would alert the experimenter of a potential problem.

If PEG and phenol red were in fact absorbed, an underestimate of water absorption calculated from those methods would result. Although commonly regarded as "unabsorbed" intestinal markers, the rat k_a for phenol red[4] and the k_a calculated from the recovery of PEG[5] were in the range of 0.002 to 0.005 min^{-1}. It appeared, therefore, that the water flux estimate calculated with the gravimetric method was most accurate, while the PEG and phenol red methods apparently underestimated water flux. Using the traditional phenol red and the proposed gravimetric methods, water flux values were calculated for an additional 10 compounds randomly selected from our database (**Table 2**). For the gravimetric method, water was absorbed (ie, flux > 0) for every compound, and water absorptive flux ranged from 36 to 106 mL/h/cm. For the phenol red method, water flux ranged from 22 µL/cm/h *secreted* to 134 µL/cm/h absorbed. Regardless of method, the average water absorptive flux with these 10 compounds by either the gravimetric or phenol red methods (74 and 65 µL/h/cm, respectively) was similar to literature values (correction method in parenthesis): 75 µL/h/cm (PEG-4000)[6] , 63 µL/hr/cm (PEG-4000)[7] , 80-110 µL/h/cm (PEG-4000)[8] , 126 µL/h/cm (inulin)[9] , 150 µL/h/cm (inulin)[10] , and 45 µL/h/cm (gravimetric)[11] .

Also shown in **Table 1** are the k_a values for the 9 studies done with Compound I. Note that the coefficient of variation (CV%, a statistic that describes the variability relative to the mean) was slightly smaller for the k_a calculated using the gravimetric method for water flux correction.

CONCLUSION

The gravimetric method therefore appeared to be as accurate as the more elaborate "nonabsorbed" marker method, and it is acceptable for SPIP studies where water flux determination is important.

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