Quantitative analysis of lab-to-lab variability in Caco-2 permeability assays

Jong Bong Lee[1], Atheer Zgair[1,2], Dhiaa A Taha[1], Xiaowei Zang[3], Leonid Kagan[3], Tae Hwan Kim[4], Min Gi Kim[4], Hwi-yeol Yun[5], Peter M Fischer[1] and Pavel Gershkovich[1]*

[1]School of Pharmacy, University of Nottingham, Nottingham, UK

[2]College of Pharmacy, University of Anbar, Anbar, Iraq

[3]Department of Pharmaceutics, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, NJ, USA

[4]School of Pharmacy, Sungkyunkwan University, Suwon, Republic of Korea

[5]College of Pharmacy, Chungnam National University, Daejeon, Republic of Korea

***Corresponding author:** Pavel Gershkovich, PhD

School of Pharmacy, Centre for Biomolecular Sciences

University of Nottingham, University Park

Nottingham, UK

NG7 2RD

Tel: +44 (0) 115 846 8014

Fax: +44 (0) 115 951 3412

Email: pavel.gershkovich@nottingham.ac.uk

ABSTRACT

In this study, Caco-2 permeability results from different laboratories were compared. Six different sets of apparent permeability coefficient (P_{app}) values reported in the literature were compared to experimental P_{app} obtained in our laboratory. The differences were assessed by determining the root mean square error (RMSE) values between the datasets, which reached levels as high as 0.581 for the training set compounds, *i.e.* ten compounds with known effective human permeability (P_{eff}). The consequences of these differences in P_{app} for prediction of oral drug absorption were demonstrated by introducing the P_{app} into the absorption and pharmacokinetics simulation software application GastroPlus™ for prediction of the fraction absorbed (F_a) in humans using calibrated "user-defined permeability models". The RMSE were calculated to assess the differences between the simulated F_a and experimental values reported in the literature. The RMSE for F_a simulated with the permeability model calibrated using experimental P_{app} from our laboratory was 0.128. When the calibration was performed using P_{app} from literature datasets, the RMSE values for F_a were higher in all cases except one. This study shows quantitative lab-to-lab variability of Caco-2 permeability results and the potential consequences this can have in the use of these results for predicting intestinal absorption of drugs.

Keywords

Drug permeability, Caco-2 permeability assay, lab-to-lab variability, prediction of oral absorption, GastroPlus™.

INTRODUCTION

The extent of gastro-intestinal absorption is one of the key properties for drugs intended for oral administration [1-6]. Of many factors that govern intestinal absorption of a compound, permeability is often tested *in vitro* by means of cell-based assays such as the Caco-2 assay [2-8]. While the Caco-2 assay is the gold standard for *in vitro* permeability assessment, interpretation of the results from such assays needs careful attention due to high lab-to-lab variability [6, 8].

This variability can be attributed to factors such as cell passage number, cell culture conditions, number of cells, cell monolayer integrity, *etc*., and can potentially mislead decisions if permeability results from different laboratories are directly compared [6]. The lab-to-lab variability itself is a known phenomenon in the field, but the extent of variability and the consequences of direct comparison of results from different laboratories have not been explicitly shown before. Therefore, the aim of this study is to compare Caco-2 permeability results between different laboratories and to evaluate potential implications of the direct use of permeability results from different laboratories for predicting oral drug absorption.

MATERIALS AND METHODS

Materials

Caco-2 cells of passage number 47 were purchased from Cell Culture Collections, Public Health England (Salisbury, UK). Dulbecco's modified eagle medium (DMEM) supplemented with GlutaMAX™, 4.5 g/L D-glucose and 25 mM 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES) was purchased from Gibco (Paisley, UK). Pravastatin was obtained from Kemprotec Ltd (Lancashire, UK). Hank's balanced salt solution (HBSS), HEPES buffer, fetal bovine serum (FBS), antipyrine, cetirizine, chlorpromazine, cimetidine,

desipramine, dexamethasone, diclofenac, furosemide, hydrochlorothiazide, ketoprofen, metoprolol, naproxen, piroxicam, propranolol, ranitidine, sildenafil, terbutaline and verapamil were purchased from Sigma (Gillingham, UK). Corning 24-well Transwell® was purchased from Fisher Scientific (Loughborough, UK). All solvents were HPLC grade or higher and all other chemicals were analytical reagent grade or higher.

Cell culture

Caco-2 cells of passage numbers 51-54 were used in the study. Caco-2 cells were passaged in 75 cm^2 cell culture flasks (Corning Inc, Corning, NY) at 1×10^4 cells/cm^2 at least twice before seeding in Transwell® plates. DMEM cell culture medium supplemented with 10% FBS and 1% penicillin-streptomycin was used and the cells were maintained at 37°C, 95% relative humidity and 5% CO_2. Cells were seeded at a density of 3.75×10^4 cells/cm^2 in the Transwell® plates. Medium was replaced on the next day following seeding and every other day thereafter for 21 days before permeability assay. Transepithelial electrical resistance (TEER) values were measured using an EVOM2 instrument (World Precision Instruments, Sarasota, FL). Caco-2 monolayers with TEER values between 280-500 Ωcm^2 were used in the permeability assay.

Caco-2 permeability assay

On the day of the experiment, the Caco-2 monolayers were washed twice with transport buffer (HBSS buffer supplemented with 10 mM HEPES and pH adjusted to 7.4 using HCl or NaOH). The cells were then allowed to equilibrate for 30 min at 37°C with the transport buffer. Donor solutions were prepared to yield 50 or 200 μM of the test compound. The assay was initiated by addition of the donor solution (300 μL) at the apical side of the monolayer. One mL of transport buffer was initially added at the basolateral side of the monolayer and 350 μL were withdrawn every 30 min up to 2 h. Fresh transport buffer (350 μL) was replaced at every

sampling time point. At the end of the assay, TEER was measured again to assess the effect of compounds on the monolayers. All experiments were performed in triplicates.

Sample analysis

Samples were analysed for pravastatin using previously reported LC-MS/MS method [9]. All other compounds were analysed using an HPLC-UV system, which consisted of a Waters 600 Pump, Waters 717 Autosampler and Waters 2996 Photodiode Array Detector. A column oven was used to maintain the column temperature at 40°C. Mobile phase was a mixture of acetonitrile and 10 mM ammonium acetate buffer with pH adjusted to 4.1 with glacial acetic acid. Specific HPLC-UV conditions are listed in Table 1.

For sample preparation, liquid-liquid extraction was applied (specific details are listed in Table 1). To 300 µL sample, 500 µL of pH modifier and 2 mL of extraction solvent were added. The samples were then vortex-mixed for 10 min and centrifuged at 1160 *g* for 10 min. The organic layer was transferred and evaporated to dryness under N_2 gas at 40°C. Reconstitution solvent (100 µL) was then added and vortex-mixed for 10 min before being transferred to HPLC vial for analysis.

Determination of apparent permeability coefficient

The apparent permeability coefficient (P_{app}) was calculated using the following equation:

$$P_{app} = (dQ/dt) \cdot (1/(A \cdot C_0))$$

where *dQ/dt* represents the steady-state flux (µmol/s), *A* represents the effective filter area of each well (cm^2) and C_0 represents the initial concentration of the donor solution (µM) [8].

In silico simulation

In silico simulation of fraction absorbed in humans (F_a) was performed using GastroPlus™ version 9.0.0007. The physicochemical properties were used as predicted by the built-in ADMET Predictor™ version 7.2.0.0. Built-in pharmacokinetic model parameters for a 30 year-old American male were used as default settings, and "Human – Physiological – Fasted" settings were applied for the gut physiology. Paracellular permeability was turned on and logD model was set as Structure-based version 6.1.

P_{app} values of compounds with known P_{eff} (training set compounds) were used as an input to calibrate the permeability model in GastroPlus™ and as a result a "user-defined permeability model" was established. The same permeability value can result in different simulation results depending on how the "user-defined permeability model" is determined. Therefore establishing a "user-defined permeability model" represents calibrating the permeability model. In other words, the "user-defined permeability model" plays a role as a calibration curve that can correlate P_{app} values to P_{eff} values. A detailed description of the "user-defined permeability model" in GastroPlusTM could be found in the Supplementary material. The training set compounds included antipyrine, furosemide, hydrochlorothiazide, ketoprofen, metoprolol, naproxen, propranolol, ranitidine, terbutaline and verapamil. P_{app} values from our laboratory and also the results from six different sets of literature values were used to produce seven "user-defined permeability models". Log-linear model was selected for all datasets as it was the one suggested by GastroPlus™ after solving the correlation for all datasets.

The experimental P_{app} values of application set compounds (desipramine, dexamethasone, sildenafil, chlorpromazine, diclofenac, piroxicam, cetirizine, cimetidine and pravastatin)

obtained from our laboratory were then applied for F_a simulation in GastroPlus™. For each compound, all input parameters were fixed and only the "user-defined permeability model" was changed for each simulation. Each P_{app} value of the application set compounds obtained from this study was applied to the seven different "user-defined permeability models" and human effective permeability (P_{eff}) values were separately predicted. These P_{eff} values were then used to simulate seven different F_a values for each compound. All other physicochemical input parameters except permeability were used as predicted by the ADMET Predictor™. Other input parameters such as particle size, precipitation time, *etc.*, were used as provided by default. The dose and formulation used for simulation are shown in Table 2.

Statistical analysis

Root mean square error (RMSE) was calculated to assess the discrepancies between datasets. RMSE was normalised by the range of each dataset so that it is always between 0-1.

RESULTS AND DISCUSSION

Permeability assay

The P_{app} values obtained from the permeability study in our laboratory are listed in Table 3. Human P_{eff} or F_a values plotted against Caco-2 P_{app} values from our laboratory are shown in Figure 1. These trends in Figure 1 are in agreement with previously reported results [1-4, 7]. However, substantial differences were found across the datasets from different laboratories for absolute P_{app} values for each individual compound (Table 3). This was demonstrated by the RMSE value, which was as high as 0.581 between the P_{app} values in our laboratory and Irvine *et al.* In this particular case, the P_{app} of antipyrine was different by as much as 13.3-fold. Therefore it is clear that Caco-2 permeability results (P_{app}) should not be directly compared across different laboratories.

Simulation of F_a

In order to demonstrate the potential consequence of directly comparing P_{app} values across laboratories, F_a was simulated using GastroPlus™ for the application set compounds (Table 4). The simulation was performed by applying the P_{app} values obtained from our laboratory to permeability models calibrated with different datasets. The RMSE values were calculated to assess the differences between the simulated F_a values and F_a values reported in the literature. The RMSE for F_a of the application set compounds simulated after calibration using P_{app} of the training set compounds from our laboratory was 0.128. When calibration using P_{app} of the training set compounds from other literature datasets were applied, the RMSE values for F_a across all of the application set compounds were higher in all except one case (0.119-0.275). Based on these high RMSE values, it can be seen that direct comparison of Caco-2 permeability results measured experimentally to those reported by a different laboratory can mislead interpretation and the subsequent predictions.

The RMSE values were further analysed according to biopharmaceutical classification system (BCS) classes (Figure 2). BCS class 3 compounds showed higher RMSE values than class 1 or 2, indicating that permeability-limited compounds can be more sensitive to the lab-to-lab variability. Additionally, it should be stressed that P_{app} values of known compounds (such as those in the training set) have to be evaluated before any permeability assessment can be made from a particular experimental setting. The literature dataset of Kerns *et al*. was an exception, where the RMSE was lower (0.119) than for the experimental results obtained in our laboratory.

In conclusion, Caco-2 cell permeability results for the same compounds can differ substantially between different laboratories. This variability can be caused by multiple factors related to the experimental setup of the assay including passage numbers, cell seeding density, monolayer

formation period, TEER achieved and transport buffer used. Specific experimental setups used in different laboratories are summarised in Table 5. The experimental differences in permeability can be critical, especially when it comes to application of these results, such as prediction of F_a. This study shows quantitative lab-to-lab variability of Caco-2 permeability results and the potential consequences this can have in application of these results in predicting intestinal absorption of drugs.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Prof. Ian Dryden for his advice on statistical analysis in this study, and Paloma Benito-Gallo for help with bioanalysis. GastroPlus™ software was kindly provided by Simulations Plus, Inc, Lancaster, California, USA.

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FIGURE CAPTIONS

Figure 1. Correlation between Caco-2 P_{app} values from experimental results and human P_{eff} values from the literature (**A**) and human F_a values from literature (**B**). P_{eff} values are from reference [7] and F_a values are from references [1-4, 10-13].

Figure 2. Root mean square error (RMSE) for simulated F_a values of application set compounds belonging to different BCS classes.

Table 1. Analytical methods including HPLC-UV and liquid-liquid extraction procedures for studied compounds

	HPLC-UV conditions						Liquid-liquid extraction conditions			
	Mobile phase		Flow rate (mL/min)	Injection volume (µL)	Column[b]	UV wavelength (nm)	pH modifier	Extraction solvent[c]	Reconstitution solvent[d]	Internal standard
Compounds	Acetonitrile	Buffer[a]								
Antipyrine	40%	60%	0.4	60	C18	243	-	DCM	40%	Verapamil
Cetirizine	50%	50%	0.5	60	C18	229	-	DCM	40%	Chlorpromazine
Chlorpromazine	50%	50%	0.5	60	C18	254	0.1 M NaOH	MTBE	40%	Verapamil
Cimetidine	60%	40%	0.3	50	CN	221	-	MTBE	40%	Verapamil
Desipramine	50%	50%	0.5	60	C18	252	-	MTBE	40%	Dexamethasone
Dexamethasone	50%	50%	0.5	60	C18	240	0.1 M HCl	MTBE	40%	Ketoprofen
Diclofenac	60%	40%	0.5	60	C18	278	0.1 M HCl	MTBE	40%	Dexamethasone
Furosemide	40%	60%	0.4	60	C18	229	0.1 M HCl	MTBE	20%	Dexamethasone
Hydrochlorothiazide	40%	60%	0.4	60	C18	271	-	MTBE	20%	Dexamethasone
Ketoprofen	50%	50%	0.5	60	C18	254	0.1 M HCl	MTBE	40%	Dexamethasone
Metoprolol	70%	30%	0.5	50	CN	223	0.1 M NaOH	EA	20%	Atenolol
Naproxen	50%	50%	0.5	60	C18	229	0.1 M HCl	MTBE	40%	Dexamethasone
Piroxicam	50%	50%	0.5	60	C18	277	0.1 M HCl	MTBE	40%	Furosemide
Propranolol	40%	60%	0.5	60	C18	229	0.1 M NaOH	MTBE	40%	Dexamethasone
Ranitidine	70%	30%	0.3	50	CN	227	0.1 M NaOH	DCM	40%	Verapamil
Sildenafil	40%	60%	0.5	60	C18	222	-	MTBE	40%	Verapamil
Terbutaline	70%	30%	0.3	50	CN	278	0.1 M NaOH	EA	20%	Metoprolol
Verapamil	40%	60%	0.5	60	C18	278	-	MTBE	40%	Desipramine

[a] The buffer used was 10 mM ammonium acetate, pH 4.1

[b] HPLC columns were as follows: C18, Gemini C18 4.6 × 250 mm, 5 µm particle size; CN, Luna CN 4.6 × 150 mm, 5 µm particle size

[c] Extraction solvents were as follows: DCM, dichloromethane; MTBE, methyl-*tert*-butyl ether; EA, ethyl acetate

[d] Reconstitution solvents are expressed as % of acetonitrile in water

Table 2. Dose and formulation input parameters of application set compounds for GastroPlus™ simulation

Compounds	Dose (mg)	Formulation
Desipramine	50	Tablet
Dexamethasone	1.5	Tablet
Sildenafil	50	Tablet
Chlorpromazine	50	Tablet
Diclofenac	50	Tablet
Piroxicam	20	Capsule
Cetirizine	10	Tablet
Cimetidine	100	Tablet
Pravastatin	40	Tablet

Table 3. P_{app} values of training set compounds from our laboratory and six different literature datasets

Compounds	Apparent permeability coefficient (P_{app}, × 10^{-6} cm/s)						
	Our laboratory	**Alsenz *et al*[a]**	**Irvine *et al*[a]**	**Li *et al*[a]**	**Zhu *et al*[a]**	**Skolnik *et al*[a]**	**Kerns *et al*[a]**
Antipyrine	11.32	54.3	150	35.7	28.2	-	12
Furosemide	0.25	0.31	0.14	1.3	0.12	1.29	0.086
Hydrochlorothiazide	0.37	0.42	0.92	1.5	0.51	1.81	0.75
Ketoprofen	10.53	24.36	93	34.7	-	18.49	20
Metoprolol	8.19	31.77	140	33.2	23.7	17.74	2.3
Naproxen	12.71	53.07	-	33.8	39.5	31.07	28
Propranolol	11.28	47.2	110	39.4	41.9	21.29	3.3
Ranitidine	0.37	0.67	-	2.1	0.49	2.51	0.47
Terbutaline	0.27	1.71	0.41	0.8	0.38	2.38	-
Verapamil	9.67	44.67	-	45.7	-	22.68	2.4
RMSE[b]	-	0.480	0.581	0.459	0.395	0.301	0.260

[a] Values from references [1-5, 7]

[b] Root mean square error between each set of P_{app} values and experimental results from our laboratory

Table 4. Caco-2 P_{app} values and simulated F_a values of application set compounds

Compounds	Caco-2 P_{app} ($\times 10^{-6}$ cm/s)[a]	Intestinal absorption (F_a, %)							
		Our laboratory[b]	Alsenz *et al.*[b]	Irvine *et al.*[b]	Li *et al.*[b]	Zhu *et al.*[b]	Skolnik *et al.*[b]	Kerns *et al.*[b]	Literature values[c]
Biopharmaceutics classification system class 1									
Desipramine	8.55	100.0	98.2	88.7	95.8	98.8	97.8	100.0	100
Dexamethasone	4.14	99.7	94.9	88.6	88.4	97.0	85.0	99.8	100
Sildenafil	22.68	100.0	99.9	98.5	99.9	99.9	100.0	100.0	92
RMSE		0.046	0.055	0.100	0.084	0.049	0.099	0.046	
Biopharmaceutics classification system class 2									
Chlorpromazine	3.7	100.0	98.3	94.2	92.8	99.3	84.9	100.0	100
Diclofenac	13.28	100.0	99.8	98.3	99.7	99.9	99.9	100.0	100
Piroxicam	11.45	99.9	98.2	91.5	97.1	98.5	98.8	99.9	100
RMSE		0.001	0.014	0.060	0.045	0.009	0.087	0.001	
Biopharmaceutics classification system class 3									
Cetirizine	1.14	87.0	59.7	58.2	36.3	74.2	16.6	93.6	60
Cimetidine	0.95	41.3	12.2	12.3	6.3	21.1	2.8	60.0	60
Pravastatin	0.14	15.8	9.8	16.4	3.3	19.3	0.2	24.7	34
RMSE		0.217	0.309	0.293	0.382	0.254	0.458	0.201	
Overall RMSE[d]	-	0.128	0.182	0.182	0.228	0.149	0.275	0.119	-

[a] Values experimentally obtained from current study`

[b] GastroPlus™ simulated F_a values based on user-defined permeability models established using datasets from our laboratory, Alsenz *et al*, Irvine *et al*, Li *et al*, Zhu *et al*, Skolnik *et al* and Kerns *et al*, respectively

[c] Experimental values of intestinal absorption in humans from references [1-4, 10-13]

[d] Root mean square error between each set of F_a values and literature values

Table 5. Different experimental settings used in different laboratories for Caco-2 permeability assays

	Passage number	Seeding density ($\times 10^4$ cells/cm^2)	Monolayer formation (days)	TEER ($\Omega \cdot$cm^2)	Transport buffer	
					Apical	Basolateral
Our laboratory	51-54	3.75	21	280-500	HBSS + 10 mM HEPES, pH 7.4	
Alsenz *et al*	103-112	15.625	7	-	40 mM Bis-Tris/120 mM Tris-base buffer, pH 7.4	
Irvine *et al*	31-42	6.3	21-25	>230	HBSS + 10 mM HEPES, pH 7.4	
Li *et al*	32-70	6	21	300-540	DMEM	
Skolnik *et al*	23-37	-	19-23	300-400	HBSS + 10 mM HEPES, pH 7.4	
Kerns *et al*	26-40	-	21-25	-	HBSS + HEPES, pH 6.0	HBSS + HEPES, pH 7.0

Supplementary material to:

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[1]School of Pharmacy, University of Nottingham, Nottingham, UK

[2]College of Pharmacy, University of Anbar, Anbar, Iraq

[3]Department of Pharmaceutics, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, NJ, USA

[4]School of Pharmacy, Sungkyunkwan University, Suwon, Republic of Korea

[5]College of Pharmacy, Chungnam National University, Daejeon, Republic of Korea

***Corresponding author:** Pavel Gershkovich, PhD

School of Pharmacy, Centre for Biomolecular Sciences

University of Nottingham, University Park

Nottingham, UK

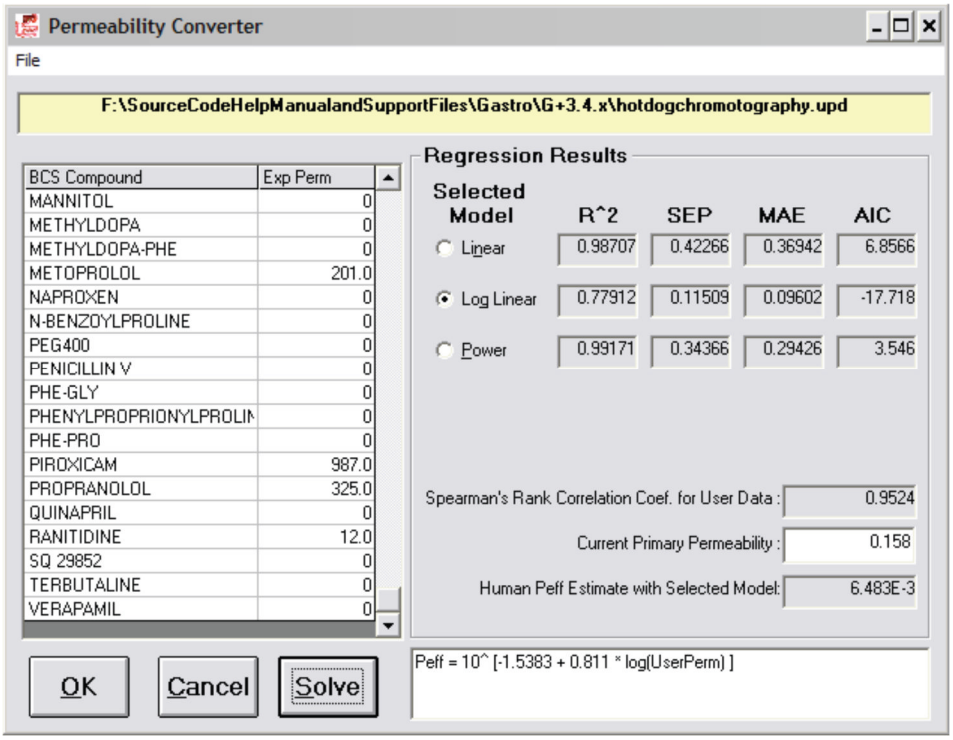
NG7 2RD

Tel: +44 (0) 115 846 8014

Fax: +44 (0) 115 951 3412

Email: pavel.gershkovich@nottingham.ac.uk

In GastroPlus™, P_{app} values of compounds with known P_{eff} can be given as input to establish a "user-defined permeability model". An example of a "user-defined permeability model" setup is shown in Supplementary Figure 1. GastroPlus™ provides a list of compounds for which the software has known values of P_{eff} (these P_{eff} values are not shown to the users). When the user inputs P_{app} values of these compounds, GastroPlus™ performs internal correlation between the input P_{app} values and their P_{eff} values. The users are then informed of the regression results of the internal correlation. The P_{app} value of the compound of interest can then be applied to this "user-defined permeability model" to give an estimate of the P_{eff} value. Therefore, establishing a "user-defined permeability model" represents calibration of the permeability model in GastroPlus™. Most importantly, same permeability value can result in different simulation results depending on how the "user-defined permeability model" is determined. In this study, same P_{app} values of application set compounds have been applied to different "user-defined permeability models" established by P_{app} values of training set compounds from different laboratories.



Supplementary Figure 1. Establishment of a "user-defined permeability model" in GastroPlusTM. The "BCS Compound" refers to the list of reference compounds provided by GastroPlusTM. The P_{app} values of these reference compounds are given as input in the "Exp Perm" column. Following the internal correlation process (initiated by clicking the "Solve" button), regression results are shown for the correlation. The user can select the models between "Linear", "Log Linear" or "Power", after analysing the parameters of each model. Following this process, the user inputs the P_{app} value for the compound of interest in the "Current Primary Permeability" box. After all these processes, the P_{eff} value estimated by the "user-defined permeability model" will be given in the "Human P_{eff} Estimate with Selected Model" box. Image is adopted from GastroPlus™ manual (August 2013 version).