Parallel Artificial Membrane Permeability Assay (PAMPA) - Is it Better than Caco-2 for Human Passive Permeability Prediction?

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Abstract: This review proposes a statistical and qualitative comparison between the main techniques derived from the original PAMPA method and experimental results obtained from Caco-2 and human jejunum. Among them, PAMPA techniques variations developed by some of the most renowned authors on the subject. The comparison is made from 16 common structurally diverse molecules, taking into account mainly membrane lipid composition and incubation conditions. A BCS classification prediction of the studied molecules was also possible to make. Finally, it was possible to raise and prove important previous hypotheses, aside from pointing out the best PAMPA model to predict human data.

Keywords: PAMPA, UWL, lipid composition, artificial membrane, BCS.

1. INTRODUCTION

The Biopharmaceutical Classification System (BCS), based on permeability and solubility properties of drugs has been proposed as a tool for developing new pharmaceutical formulations, with or without new drug molecules, aiming to assist in predicting its availability *in vivo*. According to the BCS, certain drugs can be considered for biowaivers, i.e. approving the product based on its *in vitro* dissolution rather than requiring bioequivalence studies in human subjects. As a result, unnecessary human experiments can be avoided and the costs of developing generic products can be significantly lowered [1]. To classify a drug according to the BCS, drug solubility, dose and permeability must be known. Therefore, accessing permeability is a crucial step in determining drug´s site of administration.

Undoubtedly, there is a great need to implement high throughput and low cost models, which must have a good correlation with the permeability and absorption characteristics *in vivo*. Two of the most popular *in vitro* absorption/permeability models used today involve Caco-2 cells or Parallel Artificial Membrane Permeability Assays (PAMPA). Among the *in vitro* systems employed to predict oral bioavailability in humans, Caco-2 monolayers have been recognized as the best model in terms of throughput and reliability [2-8]. Even though this model expresses some important drug transporters and cell tight junctions, its use as a high throughput tool is limited by a long cell growth cycle and high implementation costs [9-12]. For that reason, artificial membrane has been investigated as an alternative model for GI membrane simulation [13-18].

The Parallel Artificial Membrane Permeability Assay (PAMPA), first introduced by Kansy *et al.* [19] offers a high throughput approach to measure artificial membrane permeability and to assess drug absorption potential. Since the majority of drugs are absorbed primarily or partially through passive transport, the rate of permeation through a simple artificial membrane, which mimics passive transcellular transport, is likely to provide a good indication of a drug's absorption potential [15].

PAMPA was developed as a rapid, 96-well plate technology-based *in vitro* system for the evaluation of passive transcellular permeability [19]. Phosphatidylcholine (PC) has been used as a membrane constituent in the original Kansy's PAMPA system [12]. PAMPA studies are considered to be easier, faster and much less expensive than those involving Caco-2 cells. Indeed, a reliable numerical comparison between Caco-2 and PAMPA, based on a comparative study of rat in situ intestinal permeability of 17 fluoroquinolones resulted in an equally well prediction of *in vivo* data between PAMPA and Caco-2 [21].

One shortcoming of many *in vitro* studies examining drug permeability is that it lacks for standardization of protocols and many of the available literature on these important properties are not reliable or conclusive. In this regard, experimental attention to buffer pH, the use of serum proteins and co-solvents, the influence of the unstirred water layer and lipid composition are sometimes overlooked. This review will comment on the assay details of several PAMPA methods in terms of incubation conditions (pH and unstirred water layer) and it will focus mainly on lipid composition, which will be evaluated using the same molecules and compared with benchmark results using cells of Caco-2 and human jejunum. Furthermore, some suggestions for standardization of future trials will be made.

2. GENERAL ASSAY CONDITIONS

There are several parameters which may affect PAMPA results. In the donor compartment several authors applied different co-solvents, which make very low soluble com-

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pounds measurable [22-25]. For blood transport simulation, surfactant compounds were suggested by Avdeef and coworkers [26] in the acceptor phase, as they are suggested to be able to keep low free compound concentrations (mostly in case of cationic charged forms).

The pH of both phases has a huge importance in case of ionizable compounds, as the uncharged form has 3-4 magnitudes higher permeability compared to charged form. For the reason of pH partition hypothesis, the effective permeability (Pe) depends on the applied pH in case of ionizable molecules. For that reason, once the pKa value is known, it is possible to correct the results for unstirred water layer (UWL) effect and to calculate the intrinsic permeability (the permeability of the uncharged form) [27]. The acceptor pH is usually physiological blood pH, while the donor phase pH can be varied in wide range. For the UWL correction either multi pH experiments should be done or a well defined single pH measurement can be performed as described by Avdeef *et al.* (pKa^{flux}) [27].

Although some authors have early considered the porosity ratio to calculate the membrane accessible filter area [28,29], Nielsen and Avdeef [30] incorporated the apparent porosity value, εa, into the PAMPA models, taking into account the existence of an increase in porosity of the filter due to an excess of lipid impregnation. This amelioration allowed the PAMPA results obtained from different filter porosity and different amounts of lipids to be compared on the same scale [30].

2.1. Unstirred Water Layer - When it is a Real Limit Factor

According to Thomson *et al*. [31], the Unstirred Water Layer (UWL) or Aqueous Boundary Layer (ABL) is a more or less stagnant layer of water, mucus, and glycocalyx adjacent to the intestinal wall that is created by incomplete mixing of the luminal contents near the intestinal mucosal surface and can function as a barrier of absorption. The same effect appears in various permeability measurements like black lipid membrane models [32,33], artificial membrane models [23] *in vitro* cellular models [34,35], rat intestine [36,37], and human jejunum [38]. The thickness of the UWL has been estimated to be 1462 μm in PAMPA [22], 1544 μm in CaCo-2 [34], and 30-100 μm in the gastrointestinal tract [38].

In 2001, Wohnsland and Faller [28] showed that the permeability of propranolol was 40% less than that of metoprolol, even though propranolol being 40 times more lipophilic than metoprolol, which proves that, for highly lipophilic compounds, the rate-limiting barrier to transport is the UWL. In the literature, numerous techniques have been published to lower the effect of UWL [22,28,29,39]. Avdeef *et al*. [22] presented a new type of magnetic plate stirrer, which is able to rotate small orbital stir disk in every single well and reduce the thickness of the UWL till 13 μm in some cases. Applying the consideration of Adson *et al*. [40], they also stated that orbital plate shakers are unable to stir the bottom (donor) phase because the effect is dampened by the confined space over this plate [22]. Three possible calculations have been described for the correction of UWL. The traditional way has been to perform the Caco-2 assay at several different stirring speeds, in the range of 100-1000 rpm, besides from applying a hydrodynamic equation to extract cellular from apparent permeability coefficients [34,40,41]. Another technique, which is an extension of the Gutknecht procedure [33], called the pKa^{flux} method, has been described by Ruell *et al*. [29], and applies the pH partition hypothesis. The third applies results obtained from lipid-free PAMPA experiments as UWL limit [23].

Despite the precise description of the importance of UWL by lot of authors [22,27,28,30,42] some authors have obtained acceptable results even disregarding the UWL effect. Investigation of the unstirred assays incubation parameters show that some of them [20] apply higher temperature, which may affect the UWL thickness, while other uses orbital plate shakers [15] which can have a slight UWL thickness decreasing effect. The reason for the good results among unstirred assays also can be explained by the pH of the acceptor and donor compartments. Regarding the pH partition hypothesis, at pHs where the molecules are mostly ionized and the effective permeability is low, the permeability through the membrane is the rate-limiting step of the process [27]. As numerous drugs are weak acids or weak bases, at the most commonly applied pH (around 6.5) they are in their charged form without being rate-limited by the UWL. If these results are compared to data obtained from Caco-2 experiments at the same pH without UWL correction or human jejunum data, the found correlation can be excellent.

In 2009, Scherrer [43] published a paper in which he presumed that the so-called UWL phenomenon described above is not (or not only) because of a sticky water layer. He suggested a theory where the pKa^{flux} is the pKa of the molecule in the transition state, which can be described by a state along the molecules path to the membrane, from which it is as likely to continue on, as it is to return to the donor phase. Along this path the UWL is not supposed to exist [43]. The suggestion described above is based on facts proved on the field of octanol-water partitioning (especially ion-pair partitioning), but not on the field of membrane permeability. As the UWL effect is widely described in all kinds of permeability measurements, the sticky water layer still looks to be responsible for the UWL phenomenon, though the above mentioned theory needs further investigations.

It can be concluded that the UWL effect is a phenomenon which can be treated easily using the appropriate experimental parameters (multi pH, stirring) and calculations. Treating the measured data can improve the quality of the results, especially if a differentiation should be made among high permeable drugs. Controversially, a reasonable correlation have been achieved by some authors [20,44,45] without UWL correction, in which cases the above mentioned criteria may give a possible explanation.

2.2. Lipid Composition

Early attempts have suggested that the lipid composition in the intestinal membrane could be a factor to regulate the fluidity of the membrane and thereby affect the transcellular transport characteristics [46,47]. Studies with rodent small

intestine have indicated that phosphatidylcholine and phosphatidylethanolamine account for 60-70% of the total composition of intestinal membrane. Furthermore, fluidity is supposed to be controlled to a great extend by the degree of unsaturation of acyl chains and the relative proportion of cholesterol [48].

During PAMPA experiments, useful considerations on lipid composition impacts could be pointed out. Seo *et al*. [49] investigated the role of PAMPA lipid composition on the permeability of five model compounds and their results indicated that compound permeability across PAMPA differed in their sensitivity to membrane lipid composition, as compounds with appreciable permeability (i.e. at least $0.2\times10-6$ cm/s) were possibly sensitive to membrane fluidity and apparent ion pair effects. These results are compatible with Carrara and co workers [50] findings, who performed studies using a data set of 19 compounds and a validation set of 15 compounds and showed that permeability is mostly dependent on the percentage of dodecane and the effect of phospholipids is relevant only for compounds with a medium permeability value (50–100 nm/s). Furthermore, compound permeability was lowest across the phosphatidylcholines, which is consistent with this phospholipid exhibiting relatively high membrane rigidity. In contrast to results from phosphatidylethanolamines and phosphatidylserines, acyl chain unsaturation had no effect on permeability across phosphatidylcholines [49]. Corti *et al*. [51] studied the variations in the lipidic phase and its effect in drug permeability using a n-octanol–Lipoid® E 80-cholesterol ternary mixture. Likely previous studies, it was hypothesized that higher amounts of lipid phase impregnated into the support corresponded to lower drug Papp values by the presence of a thicker diffusion layer which reduces the drug permeation rate [51].

In order to separate active and passive components of cellular permeability, Dagenais and co-workers developed the in combo PAMPA, where a hybrid combination of in silico and *in vitro* methods was made [52]. Another improvement has recently drawn considerable attention. A novel procedure for preparation of artificial membrane suggested the formation of a lipid/oil/lipid tri-layer and showed to reduce the mass retention of highly retained compounds reported in previous studies. Moreover, the lipid/oil/lipid layer - which can also be called as Tri-layer membrane PAMPA- proved to reduce the amount of compounds retention in the membrane, by replacing long chain solvents by volatile solvents [44]

In the present work a comparison between different variations of the PAMPA original method was made, in an attempt to find the model which best correlates with human results and, thus, to point out the method of choice for future experiments. The choice of methods for comparison in this study was based on the most referenced authors in the subject who used the same molecules in their studies.

3. PAMPA DATA EVALUATION

The following PAMPA methods results were obtained from literature: a) Tri-layer membrane PAMPA [44]; b) Biomimetic PAMPA [20]; c) 10% Lecithin PAMPA [53]; d) 1% Lecithin PAMPA [15]; and e) in combo PAMPA [52]. Each assay condition varies mainly on its lipid composition and their detailed description can be found at Table **1**. Furthermore, each particular technique was compared with human jejunum and Caco-2 experimental data found in literature and the results are shown at Table **2**.

Firstly, the permeability results obtained from the different PAMPA methods (Table **2**) were statistically compared to the standard human jejunum effective permeability (P_{eff})

Table 1. PAMPA Methods Descriptions

Method	Lipid composition	Incubation	References
Tri-layer membrane PAMPA	Hexadecane, phospholipids mixture and hexane, in a try-layer configuration	Room temperature; without agitation; 4 to 5h	[44]
Bio-Mimetic PAMPA	PC (0.8%)/PE (0.8%)/PS (0.2%)/PI (0.2%)/CHO (1.0%)/1,7-octadiene (97.0%)	30ºC; without agitation; 2 or 15h	[20]
10% Egg Lecithin PAMPA	10% lecithin in 1,9-decadiene	25ºC; without agitation; 2, 5 or 24h	[53]
1% Egg Lecithin PAMPA	1% egg lecithin in n-dodecane	Room Temperature; gentle circular shaking; 2h	[15]
in combo PAMPA	20% (w/v) alkane solution of a lecithin mixture containing an excess of negatively charged constituents	Room temperature, individual magnetic stirring, 30 min or 15h	[52]

PC = phosphatidylcholine.
PE=phosphatidylethanolamine.
PS = phosphatidylserine.
PI = phosphatidylinositol.
CHO = cholesterol.

Table 2. Permeability Results from Different PAMPA Methods (Results are Expressed in 10^{-6}cm/s) Considering 16 Structurally Different Drugs

Compounds	Perm. Class.[a]	logD pH 7.4[b]	Human jejunum Peff ($*10^6$ cm/s)[c]	Caco-2 Peff ($*10^6$ cm/s)[d]	Peff ($*10^6$ cm/s)				
					In combo PAMPA[e]	Tri-layer membrane PAMPA[f]	10% Egg Lecithin PAMPA[g]	Bio-mimetic PAMPA[h]	1% Egg Lecithin PAMPA[d]
Amiloride	H	-0.86	162.18		4.47	0.08		32.36	0.00
Antipyrine		0.56	446.68	28.2	10.23	7.59	2.88	181.97	20.42
Atenolol	L	-2.01	19.95	0.20	2.75	0.10		2.19	0.10
Carbamazepine	H	2.45	426.58		147.91	9.55		645.65	12.02
Cimetidine	L	0.34	30.20	0.74	3.31			5.50	0.00
Desipramine		1.38	436.52	21.6	239.88	8.71	16.98	295.12	14.45
Furosemide	L	-0.24	5.01	0.12	5.25	0.47	0.34	8.32	0.60
HTZ	L	-0.18	3.98	0.51	1.82	0.09	0.20	3.55	0.10
Ketoprofen	H	-0.11	831.76		177.83	4.17	2.82	588.84	19.05
Metoprolol	H	-0.24	128.82	23.7	4.57	4.27	7.94	67.61	3.47
Naproxen		0.09	831.76	39.5	371.54	6.03	5.01	645.65	24.55
Piroxicam		0.00	724.44	35.6	194.98	5.01	10.96	602.56	8.51
Propranolol	H	1.41	309.03	41.9	72.44		26.30	354.81	2344
Ranitidine	L	-0.53	27.54	0.49	0.87	0.45	0.89	4.27	0.50
Terbutaline		-1.35	30.20	0.38	4.79	0.47		5.25	0.10
Verapamil	H	2.51	676.08		120.23	8.91	22.91	489.78	9.77

[a]Extracted from [1].
[b]Extracted from [55].
[c] Extracted from [24,54].
[d] Extracted from [15].
[e]Extracted from [52].
[f] Extracted from [44].
[g]Extracted from [53].
[h]Extracted from [54].
HTZ = Hydrochlorothiazide.
Perm. Class. = Permeability Classification.

values, and the following logarithmic correlation coefficients (r^2) were obtained: 0.53 (10% Egg Lecithin PAMPA; 0.70 (Tri-layer membrane PAMPA); 0.72 (1% Lecithin PAMPA); 0.77 (in combo PAMPA); and 0.87 (Bio-mimetic PAMPA). Sugano *et al*. considered a prediction for paracellular pathway using Renkin function to construct their Bio-mimetic PAMPA model. The Renkin function is the dimensionless molecular sieving function for cylindrical channels. The aqueous diffusivities of charged permeants are equivalent to those of uncharged species in a medium of sufficiently high ionic strength. The product is the effective diffusion coefficient for the filter pore, which compares the compounds molecular radius with the filter pore radius [54]. This can be a possible explanation for the better correlation when compared with the other methods.

Likewise, it can be concluded that the 1% Lecithin and the Tri-layer membrane PAMPA methods can equally predict the human reference results, which proves that a more complex protocol for preparing a multi-layer system is not needed. Furthermore, due to the better prediction using the 1% Lecithin when compared to the 10% Lecithin PAMPA method, it can be suggested that an increase in the concentration of phospholipids adversely affects the human permeability prediction. This suggestion confirms the statement that an excess of lecithin can show very high membrane retention, in some cases preventing the assessment of permeability by UV spectrophotometry [27].

Comparing the correlation of the high lipophilic molecules it can be observed that the methods which correct the results for UWL [20,52] has a much better correlation with human jejunum Peff compared to those which do not corrected it. This can be explained by the observation that the uncharged forms of high lipophilic compounds are rate-limited by the aqueous boundary layer and not by their membrane permeability.

A statistical logarithmic correlation between human P_{eff} and Caco-2 resulted in a coefficient of 0.73, which shows that, although Caco-2 is suggested to reliably predict the

human data [2-8] its correlation was lower than those found in in combo [52] and Bio-mimetic PAMPA [54] methods. This finding reinforce the fact that PAMPA methods which consider both phospholipid mixture membrane impregnation and in silico approach is as good as (or better than) Caco-2 for the prediction of passive permeability from structurally diverse compounds.

Except for amiloride, the majority of the methods were able to predict the BCS classification of all compounds included in this study. Christel *et al*. [56] has suggested that amiloride has high permeability and an intermediate solubility, so they suggested that it fits in a BCS classification called V. Its fraction of absorbed dose in humans (FA%) results is also controversial between different authors, where most of them consider that amiloride has a FA% of 50 [15,28,29,44,54] and other mention a value of 80-90% [57]. For that reason, amiloride can be considered a molecule which is not easy to predict.

Finally, a correlation between both PAMPA (i.e. Bio-Mimetic PAMPA) and LogD o/w pH 7.4 and human jejunum and LogD o/w (also in pH 7.4) was calculated. The coefficients obtained were 0.36 and 0.29, respectively. These results are in disagreement with those stated by Galinis-Luciani *et al*. [58] who affirmed that PAMPA effective permeability and measured octanol-water partition coefficients at pH 7.4 correlated well. This concern was previously criticized by Avdeef and co-workers [24], who showed a study based on 237 compounds, where the regression standard deviations in PAMPA versus octanol-water partition coefficients could be as high as 1.6 log units.

CONCLUSION AND OUTLOOK

In more than 10 years past numerous different PAMPA models have been published for the intestinal absorption prediction. In this study we proved that *in vitro* models which consider a phospholipid mixture including the main lipid species from the human jejunum constitution and combined with in silico calculations are able to predict passive human jejunum permeability results better or, sometimes, equally good as Caco-2 measurements do. Because of its much lower cost and high standardization potential, PAMPA is a useful tool for early stages of drug discovery and also can be able to replace difficult and expensive Caco-2 results in passive permeability measurements. A standardization of the PAMPA methods which showed the best correlations in this work could be considered for further studies.

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