

The PAMPA Story

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PAMPA, a high-throughput technique for measuring membrane permeability of pharmaceutical research compounds, is only about three years old, but its story has roots planted in the early 1960s. It is like other stories of how scientists in pharmaceutical companies solve the problems of getting molecules that can kill bacteria in a test tube to do the same in the human body.

Today, using combinatorial chemistry to generate new molecules, in the course of a year a large company may screen a staggering number of molecules for biological activity. In the early stages of drug discovery, these tests are performed with high-throughput screening (HTS) robotic systems, which measure a candidate molecule's ability (a) to bind to a target site on an isolated enzyme or protein and cause the macromolecule to cease its undesired action, or (b) to stunt the growth or kill a colony of pathogenic cells or organisms. In the post-human genome era, as disease-related genes are discovered with accelerating frequency, thousands of additional opportunities arise to test new drug targets. A successful hit at the early stage is just the beginning in a long journey before a molecule can become a drug. A minuscule number of molecules succeed in the end.

Many fail due to problems with absorption in the intestine. The molecules work in a test tube, but in the body they just can't reach their therapeutic target sites. To get to such a site, a molecule has to permeate through many road blocks formed by cell membranes, composed of phospholipid bilayers, which are oily barriers that prevent the passage of charged or polar molecules. Laboratory test animals usually are not employed at the earliest screening stage to assess molecules for absorption efficiency. Often, cultured cells, such as Caco-2, MDCK, and HT29 [1-4], are used for this purpose. These tests can be costly and require specialized equipment and skills. With the aim of improving efficiency and lowering costs, researchers have considered other types of permeability measurements, based on artificial membranes.

Since the the topic of cultured cell-based assays has been well covered in several recent reviews and conferences, the PAMPA2002 symposium will focus on the rapidly emerging new *in vitro* technology based on the use of filter-immobilized artificial membranes, constructed of phospholipid bilayers supported on high-porosity microfilters. Such use of model membranes to assess permeability goes back at least to the early 1960s.

Mueller *et al.* [5] discovered in 1962 that when a small quantity of a phospholipid (2% wt/vol alkane solution) was carefully placed over a small hole in a thin sheet of teflon, a thin film gradually forms at the center of the hole, with excess lipid flowing towards the perimeter. Eventually, the central film turns optically black as a single bilayer lipid membrane (BLM) forms over the hole. Suitable lipids for the formation of a BLM are mostly isolated from natural sources, e.g., phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and others. Such membranes have been viewed as useful models of the more complex natural membranes.

However, a serious drawback in using BLMs as a model system is that they are extremely fragile (requiring a vibration-damping platform and a Faraday cage), and tedious to make. Using such delicate apparatus, Walter and Gutknecht [6] studied the permeation of a series of simple carboxylic acids across eggPC/decane BLMs. Intrinsic permeability coefficients, P_o , were calculated from tracer fluxes. A straight line relationship was observed between $\log P_o$ and hexadecane-water partition coefficients, $\log K_p$, for all but the smallest carboxylic acid (formic): $\log P_o = 0.90 \log K_p + 0.87$.

Using a similar BLM system, Xiang and Anderson [7] studied the pH-dependent transport of a series of α -methylene-substituted homologs of p-toluic acid. They compared the permeabilities to partition coefficients determined in

octanol-, hexadecane-, hexadecene-, and 1,9-decadiene-water systems. The poorest correlation was found with octanol. With the decadiene-water system, $\log P_o = 0.99 \log K_p - 0.17$ (r^2 0.996). Corrections for the unstirred water layer were key to these analyses.

Efforts to overcome the limitations of the fragile membranes (thought to be as delicate as soap bubbles) have evolved with the use of membrane supports, e.g., polycarbonate filters or other more porous microfilters [8].

Cools and Janssen [9] studied the effect of background salt on the permeability of warfarin through octanol-impregnated membranes (Millipore ultrafiltration filters, VSWP, 0.025 μm pores). At a pH where warfarin was in its ionized form, it was found that increasing background salt increased permeability. This observation was thought to support an ion-pair mechanism of transport of charged drugs across real biological membranes. However, current understanding of the structure of wet octanol [10], suggests that this isotropic solvent system may not be a suitable model for passive diffusion of *charged* drugs across phospholipid bilayers.

Camenisch *et al.* [11] measured the pH 7.4 permeabilities of a diverse group of drugs across octanol- and isopropylmyristate-impregnated artificial membranes (Millipore GVHP mixed cellulose ester filters, 0.22 μm pores), and compared them to permeabilities of the Caco-2 system, and octanol-water apparent partition coefficients, $\log D(\text{pH } 7.4)$. It is reasonably clear that the uncharged drug species were the passive diffusion permeants. (When the GVHP membrane was not impregnated with a lipid, the permeabilities of all the tested drugs were high and largely undifferentiated, indicating only the unstirred water layer resistance.) Over the range of lipophilicities, the curve relating the effective permeabilities, $\log P_e$, to $\log D(\text{pH } 7.4)$ was sigmoidal in shape, and only linear in the mid range. Between $\log D(\text{pH } 7.4) -2$ and 0, $\log P_e$ values correlated with the apparent partition coefficients. However, outside that range, there was no correlation between permeabilities and the octanol-water partition coefficients. At the high end, the permeabilities of very lipophilic molecules are limited by the unstirred water layer and not the membrane *per se*. At the low-permeability end, very hydrophilic molecules were observed to be more permeant than predicted by octanol, due to an unidentified mechanism.

Kansy *et al.* [12,13] from Hoffman-La Roche roused the pharmaceutical research community with a widely-read publication on the permeation of drugs across phospholipid-coated filters. Their report could not have come at a better time -just when the paradigm was shifting into screening for biopharmaceutical properties at high speeds, along side the biological screening. Their PAMPA (parallel artificial membrane permeability assay) method has attracted a lot of favorable attention, and has spurred the development of a commercial instrument. [14,15] The Roche investigators were able to relate their measured fluxes to human absorption values with a steep sigmoidal curve, much like that indicated in Caco-2 screening. The outliers in their assays were molecules known to be actively transported. Since the artificial membranes have no active transport systems and no metabolizing enzymes, the assay would not be expected to model actively transported molecules. What one sees with PAMPA is pure passive diffusion of the uncharged species. In the last twelve months, several publications have emerged, describing PAMPA-like systems. [14-23]

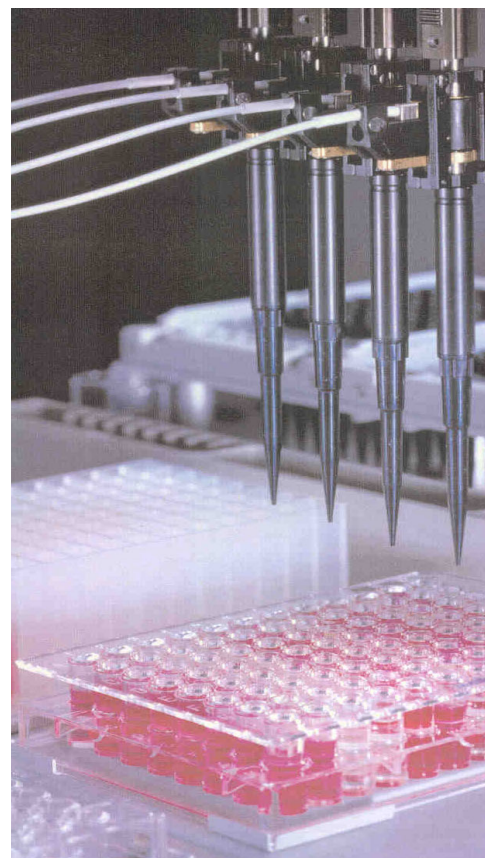


Fig. 1 The PAMPA "sandwich" formed with a 96-well microtitre plate on the bottom and a 96-well filter plate on the top. The filters separating the two plates are coated with a phospholipid-containing oil.

The system reported by Avdeef and coworkers [14,15,23] is an extension of the Roche approach, with several novel features described, including a way to assess membrane retention. In the PAMPA assay, a "sandwich" is formed (Figure 1) from a 96-well microtitre plate and a 96-well microfilter plate, such that each composite well is divided into two chambers: donor at the bottom and acceptor at the top, separated by a 125 μm -thick microfilter disc, coated with a 2% wt/vol dodecane solution of dioleoylphosphatidylcholine (DOPC), under conditions that multilamellar bilayers form inside the filter channels when the system contacts an aqueous buffer solution. The Roche group used an artificial membrane formulation consisting of egg lecithin in dodecane. [13] Thin (10 μm), low porosity (20%), polycarbonate filters [18,19], popular in Caco-2 assays, appear too fragile for high-throughput PAMPA applications; better reproducibility has been achieved with the thicker (125 μm), higher-porosity (70%), filters reported by the Roche group.

Avdeef [23] derived the iso-pH permeability equation which directly takes into account the membrane retention of a drug:

$$P_e = - \frac{2.303}{A(t-\tau_{ss})} \left(\frac{V_A V_D}{V_A + V_D} \right) \log_{10} \left[1 - \left(\frac{V_A + V_D}{(1 - R) V_D} \right) \left(\frac{C_A(t)}{C_D(0)} \right) \right] \quad (1)$$

where A = area of filter (0.3 cm^2), t = time (s), τ_{ss} = steady-state time(s), V_A and V_D are the acceptor and donor volumes (cm^3), respectively, and $C_A(t)$ and $C_D(t)$ are the measured acceptor and donor sample concentrations (mol cm^{-3}) at time t, respectively. The retention factor, R, is defined as $1 - [C_D(t) + C_A(t) \cdot V_A / V_D] / C_D(0)$, but is often reported as a percentage.

Figure 2 shows the appearance of dihydromethysticin in the acceptor well as a function of time. [15] The solid curve is a least-squares fit of the data points to eq. 1, with the parameters: $P_e = 32 \times 10^{-6} \text{ cm/s}$, $R = 42\%$, and $\tau_{ss} = 35 \text{ min}$. The membrane retention, R, is often stated as a mole percentage (%R) of the sample. Its value can at times be very high, as high as 90% for chlorpromazine and 70% for phenazopyridine, when 2% wt/vol DOPC in dodecane is used. Figure 3 shows a plot of log %R vs. the apparent octanol-water partition coefficient (at pH 7.4). It appears that retention is due to the lipophilicity of molecules. It may be a good predictor of the pharmacokinetic volume of distribution or of protein binding.

Culture-cell assays also are subject to sample retention by the monolayer. Wils *et al.* [24] reported retentions as high as 44%, whereas Sawada *et al.* [25-27] cited values as high as 89%. It is undoubtedly a common phenomenon with research compounds, which are often very lipophilic. Yet in most reported studies, the effect is ignored, it appears.

Faller and Wohnsland [18,19] developed the PAMPA assay using phospholipid-free hexadecane, supported on polycarbonate

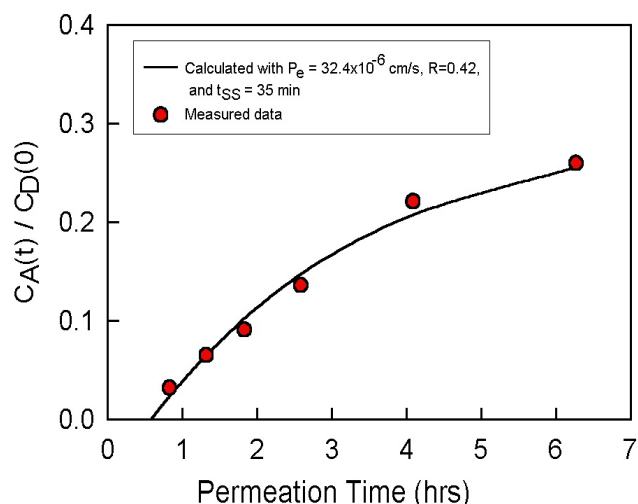
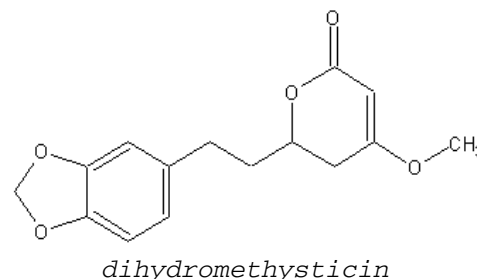


Fig. 2 Appearance of dihydromethysticin in the acceptor compartment as a function of time.

filters, and were able to demonstrated interesting predictions. Sugano and coworkers [21,22] explored the lipid model containing several different phospholipids, resembling the mixture found in reconstituted brush-border lipids, [30,31] and demonstrated improved property predictions.

The structure of the filter-immobilized artificial membranes is not known at this time. Thompson *et al.* [8] hypothesized that polycarbonate filters had a single bilayer per pore. Hennesthal and Steinem [28] using scanning force microscopy, estimated that a single bilayer spans exterior pores of porous alumina. These observations may be incomplete, as there is considerable complexity to the spontaneous process of the formation of BLMs. When 2% PC-dodecane solution is suspended in water, with water content > 40 wt%, the lipid solution takes on the inverted hexagonal structure, where the polar head groups of the PC face water channels in a cylindrical structure. [29] Such structures can alter transport properties, compared to those of normal phases. Suspensions of 2% PC-dodecane have been titrated from pH 10 down to pH 3. [Avdeef, unpublished] Along the way, at about pH 4, the pH electrode is choked by a clear gelatinous coating, suggesting that some sort of phase transition takes place then. It is particularly important in the PAMPA method that all depositions of the phospholipid be done under highly standardized procedures. Based on the observed PAMPA permeability of salicylic acid, and that observed in a BLM experiment [6], and the additivity of inverse permeabilities, it has been estimated that a permeant traverses about 100 - 300 bilayers in passing through the 125 μm phospholipid-impregnated filters. [Avdeef, unpublished] It now appears that the case for a single bilayer has not been definitively made.

The world-wide PAMPA research of the last three years has raised questions at a rate outpacing answers. The PAMPA2002 Symposium in San Francisco in July 2002 is expected to be a watershed meeting, with exciting exchange of scientific ideas surrounding the new high-throughput screening methodology, especially directing focus on its relationship to the established Caco-2 assays currently in wide use.

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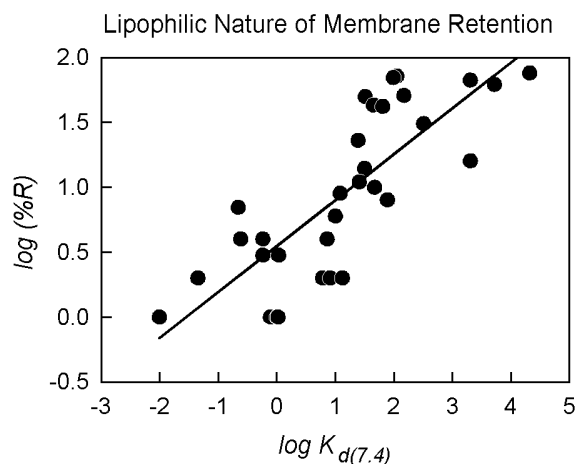


Fig. 3 Log of the %mole fraction membrane retention vs. log of the apparent octanol-water partition coefficient at pH 7.4. Microfilters were impregnated with 2% wt/vol DOPC in dodecane.

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