Development of High-Throughput PAMPA as an *In Vitro* Model of Passive Transcellular Permeation

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ABSTRACT

The absorption of orally administered compounds is largely determined by their ability to cross the gastrointestinal tract. Cell culture models can be labour intensive and limited to a narrow pH range. The majority of drugs are absorbed through passive (or partially passive transport). Assays using artificial membranes, such as PAMPA (parallel artificial membrane permeation assay) can be used as an alternative approach to assess *in vitro* transcellular passive permeation.

PAMPA avoids the complexities of active transport allowing test compounds to be ranked based on a simple permeability property alone. Using this approach differing pH values can be assessed to mimic the conditions in the gastrointestinal tract, or to overcome solubility issues. We have developed a reliable, high throughput PAMPA that measures passive permeability across an artificial hexadecane membrane. Permeation of test compound through a pre-prepared artificial membrane into the receiver well is monitored by LC-MS/MS following a 5 hr incubation period at room temperature.

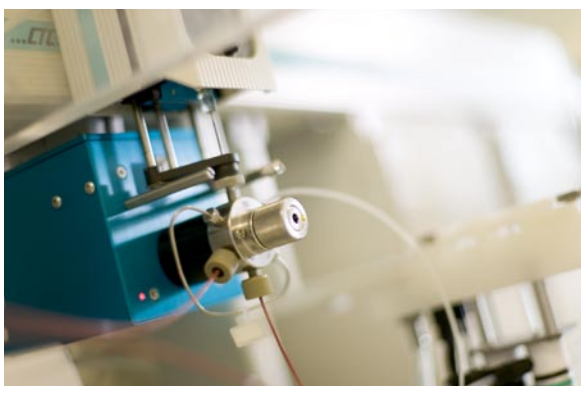
By utilising a combination of liquid handling robots, automated LC-MS/MS analysis and a tailored laboratory information management system, the procedure is highly efficient, and enables large numbers of discovery compounds to be screened rapidly and cost-effectively. PAMPA is applied early in drug discovery as a primary permeability screen to identify compounds which are anticipated to be poorly permeable *in vivo*. A valuable insight into the mechanisms of absorption can be gained using data generated in PAMPA in conjunction with data generated in cell-based permeability assays.

INTRODUCTION

With the onset of parallel lead optimisation, bringing cheaper quicker ADME screens earlier in the discovery process is considered essential. Permeability studies using artificial membranes provide benefits over cell-based assays in that no cell culture is required enabling screening to be performed without delay in a cost effective manner. Artificial membrane assays enable a wider pH range to be investigated compared to cell based assays.

The parallel artificial membrane permeability assay (PAMPA) measures permeability across an artificial membrane. This method provides an *in vitro* model for passive diffusion.
Passive diffusion is an important factor in determining transport through the gastrointestinal tract, penetration of the blood brain barrier, as well as transport across cell membranes. Permeability can also be influenced by several other mechanisms including paracellular transport and active uptake or efflux which are not assessed in PAMPA. Therefore, PAMPA provides a simplistic approach to permeability by only measuring a single mechanism. This avoids the complexities of active transport/efflux and metabolism and enables the compounds to be ranked on a single permeability property.

As part of its Cloe®Screen range of assays, Cyprotex have developed high quality PAMPA which determines permeability by quantification of the acceptor and donor concentrations after a 5 hr period using a 5 point standard curve. The use of high capacity automated pipetting system and automated data processing enables rapid screening of compounds in a cost effective manner.
By reducing the cost and increasing the speed of the assay, PAMPA can be performed at an earlier stage in drug discovery.



FIGURES AND TABLES

FIGURE 1.
Reproducibility of 13 compounds in 3 separate PAMPA experiments at pH 7 (error bars represent the standard deviation of up to 8 replicates per experiment)

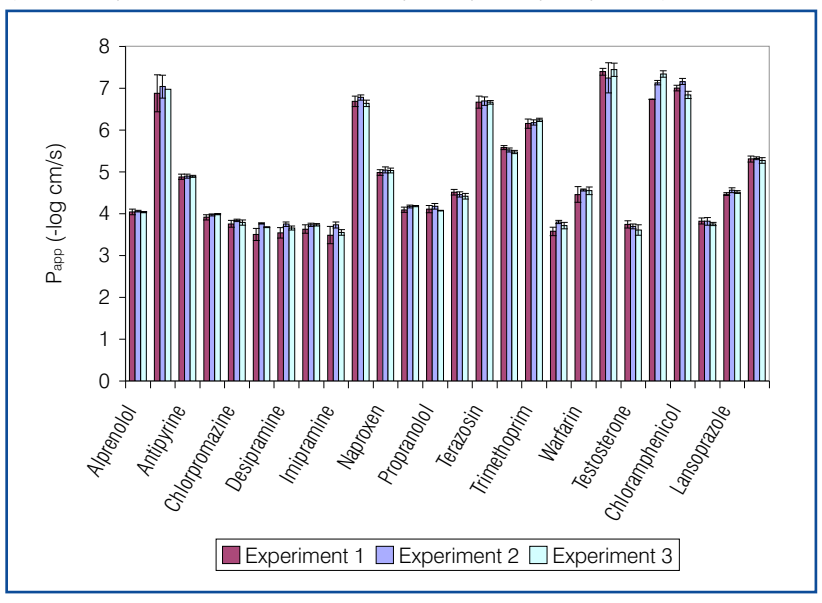


FIGURE 2.
Comparison of Cloe®Screen PAMPA data at pH 7 with third party data

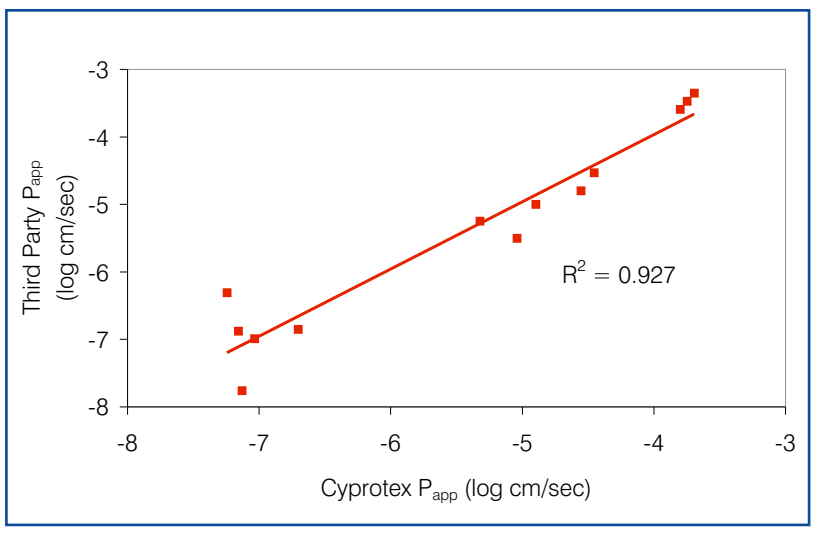


TABLE 1.
Effect of pH on PAMPA permeability for acidic and basic compounds

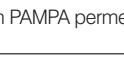
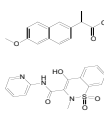
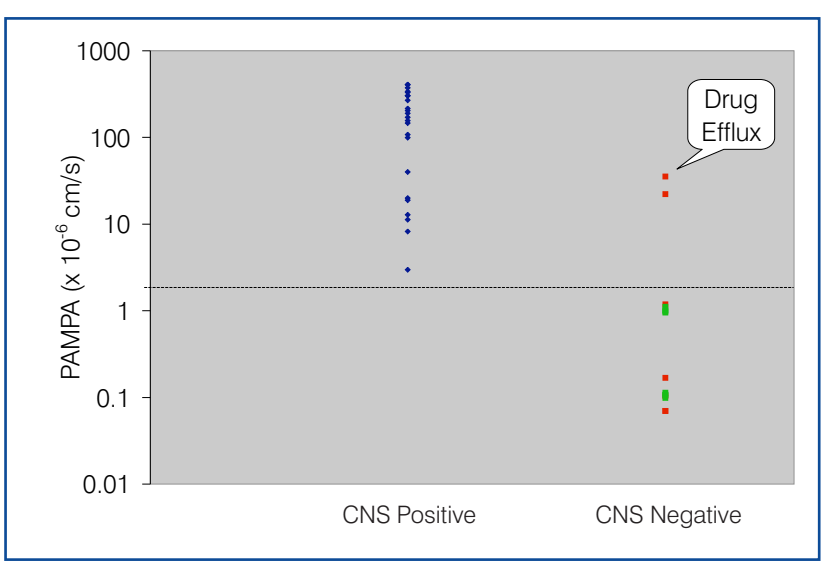
		PAMPA (x 10^{-6} cm/s)			% Fraction Absorbed
		pH 4	pH 7	pH 8	in Humans[1]
ACIDS					
Naproxen (pKa = 4.2)		281	8.82	0.807	99
Piroxicam (pKa = 5.1)		140	36.2	9.13	100
BASES					
Desipramine (pKa = 10.6)		0.475	190	180	100
Verapamil (pKa = 9.1)		3.45	220	174	100

FIGURE 3.
Classification of CNS positive or negative drugs[2,3] using PAMPA permeability at pH 7.



METHODS

1. Experimental Protocol

A solution of hexadecane in hexane is prepared (5% v/v) and an aliquot added onto the membrane of each well in the filter (donor) plate (Multiscreen® filter plate for PAMPA, Millipore). The donor plates are then allowed to dry to ensure evaporation of hexane. Buffer (pH 7 or other selected pHs) containing a final DMSO concentration of 5% is added to each well of the acceptor plates. Test compound solutions are prepared by diluting 10mM DMSO concentrates in buffer to give a final test compound concentration of 10μM (final DMSO concentration 5%). The fluorescent integrity marker Lucifer yellow is also included in the test compound solution. Test compound solutions are filtered before addition to the donor plate. The donor plate is inserted into the acceptor plate and the plates are then incubated at room temperature, in a humid environment, for 5 hours. Analytical standards are prepared from filtered test compound solutions. Test compound permeability is assessed in quadruplicate at each pH. On each plate compounds of known permeability characteristics are run as controls.

At the end of the incubation period the donor plate is removed from the acceptor plate. The donor and acceptor samples for test and control compounds are quantified by LC-MS/MS cassette analysis using a 5-point calibration with appropriate dilution of the samples. The experimental recovery is calculated from both donor and acceptor compartment concentrations. Cyprotex generic analytical conditions are used.

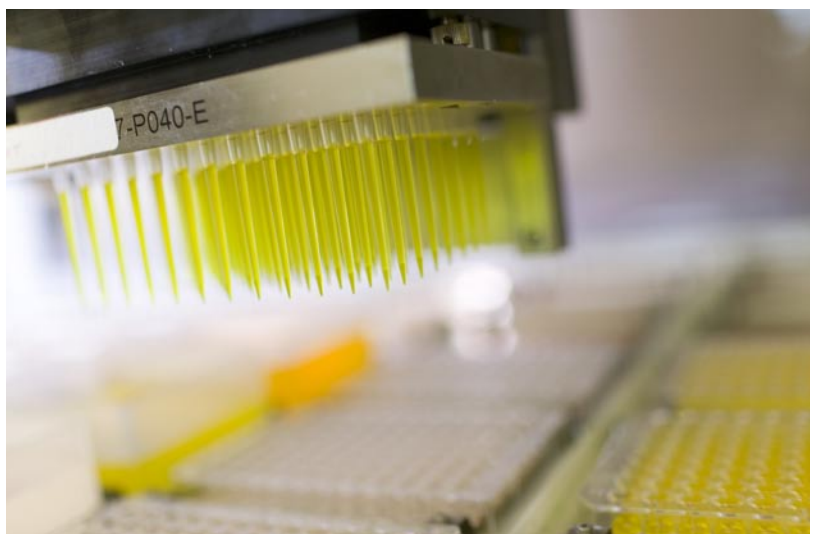
2. Data Analysis

The apparent permeability coefficient for each compound (P_{app}) is calculated from the following equation:

$$P_{app} = C \times -\ln\left(1 - \frac{[drug_{acceptor}]}{[drug_{equilibrium}]}\right)$$

$$\text{Where} \quad C = \frac{V_D \times V_A}{(V_D + V_A) \times \text{area} \times \text{time}}$$

Where V_D and V_A are the volumes of the donor and acceptor compartments, respectively, area is surface area of the membrane multiplied by the porosity and the equilibrium drug concentration is the concentration of test compound in the total volume of the donor and acceptor compartments.



We have automated the entire process so that we can analyse 48 test compounds at a time in quadruplicate plus controls, with the use of a BasePlate™ liquid handling robot (The Automation Partnership, Royston, UK), cassette LC-MS/MS analysis and a tailored laboratory-information management system – enabling the analysis of large numbers of discovery compounds in a cost effective manner.

RESULTS

Intra– and Inter-Assay Reproducibility

To assess intra– and inter-assay reproducibility, 13 compounds with a range of permeability values were selected. These compounds were replicated 8 times on the same plate, and were screened on three separate occasions. Figure 1 displays the P_{app} values for each of the three experiments with the error bars representing the standard deviation of the mean of the eight replicate wells. The P_{app} values generated showed high consistency within run and between each run of the assay. Furthermore, the detection and quantification was sensitive for a range of permeability values.

ii. Comparison with Third Party Data

Figure 2 displays the PAMPA P_{app} generated using Cloe®Screen against third party values. Cloe®Screen PAMPA uses a highly sensitive method of detection using LC-MS/MS whereas the method of detection of the third party was UV absorption. For the latter method, prolonged incubation times were required for compounds which were poorly permeable whereas the Cloe®Screen LC-MS/MS analytical method enabled a single short term incubation to be applied for all compounds. The data generated by Cloe®Screen PAMPA were highly comparable with the third party data (R2 = 0.927).

iii. Effect of pH on PAMPA Permeability

The effect of pH on PAMPA permeability was evaluated for 4 well known ionisable drugs (2 acids and 2 bases). The compounds were screened at pH 4, pH 7 and pH 8. Table 1 illustrates the important of pH and its effect on permeability. The two acidic drugs, naproxen and piroxicam, showed much higher permeability at pH 4 compared to pH 7 and pH 8. In contrast, the two basic drugs, desipramine and verapamil, were more permeable at pH 7 and pH 8 compared to pH 4. All four drugs were almost completely absorbed in humans.

iv. Classification of CNS positive and CNS negative compounds using PAMPA

A set of known marketed drugs were classified as CNS negative (15 compounds) and CNS positive (23 compounds), based on their ability to cross the blood brain barrier. The permeability of these drugs was assessed in the Cloe®Screen PAMPA. It was evident from the data that the CNS positive drugs tended to exhibit higher P_{app} values than the CNS negative drugs. The cut-off for the classification appeared to be a P_{app} of 2 x 10^{-6} cm/s. None of the CNS positive drugs and only 2 out of 15 CNS negative drugs were misclassified. The CNS negative drugs which were misclassified were quinidine and vinblastine, both of which undergo efflux by p-glycoprotein.

CONCLUSIONS

PAMPA has the following advantages over cell based permeability assays;

- No requirement for cell culture facilities resulting in a significant reduction in cost and time.
- Cell based assays may have metabolising enzymes present which may produce misleading data.
- PAMPA avoids the complexities of active transport allowing test compounds to be ranked based on a simple permeability property alone.
- A wide range of pH values can be assessed in PAMPA to mimic the conditions in the gastrointestinal tract, or to overcome solubility issues.

Cyprotex offers a rapid and cost effective method for assessing PAMPA permeability at multiple pH values. The method has been developed using a high quality protocol and displays good inter and intra-assay reproducibility. The data generated has been further validated by comparison will a third party and provides an excellent correlation.

There are several applications for PAMPA.

1. Firstly, PAMPA is commonly used as a very early screen to identify compounds which are poorly permeable. This is often performed as a filter screen, and compounds which fall below a pre-determined P_{app} in the PAMPA are rejected.
2. Secondly, PAMPA is used as a model of transcellular permeability in the gastro-intestinal tract following oral administration[4]. However, the main site of drug absorption is in the small intestine where the pH varies from acidic to neutral and slightly basic. According to the pH partition hypothesis, for ionisable compounds the ability to cross a lipid membrane is dependent on the molecule being in its non-charged state. Therefore, the pH selected for the permeability studies is critical for evaluating drug absorption, and relying on data from a single pH can be misleading. It is recommended that multiple pH values are assessed, or the pH selected is based on the physicochemical properties of the compound.
3. Thirdly, PAMPA shows a relationship with brain penetration for compounds which are not subject to active transport. PAMPA may, therefore, be used as an early screen for passive BBB permeation. Other cell based permeability assays which contain the relevant active transporters can then be used as a secondary screen to confirm the PAMPA result.

By combining the data generated in PAMPA with cell-based permeability assays, a valuable insight into the mechanisms of absorption can be gained.

REFERENCES

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