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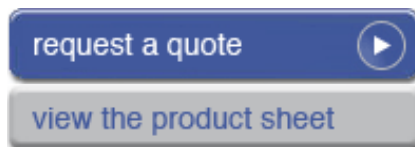
## Caco-2 permeability assay

Understand the suitability of your compound for oral dosing by using our Caco-2 permeability assay to predict human intestinal permeability and to investigate drug efflux.

Caco-2 permeability is included in our portfolio of *in vitro* ADME screening services. Cyprotex deliver consistent, high quality data with cost-efficiency that comes from a highly automated approach.

### Caco-2 permeability assay to investigate intestinal permeability

- Cyprotex's Caco-2 Permeability assay uses an established method for predicting the *in vivo* absorption of drugs across the gut wall by measuring the rate of transport of a compound across the Caco-2 cell line.
- The Caco-2 cell line is derived from a human colon carcinoma. The cells have characteristics that resemble intestinal epithelial cells such as the formation of a polarized monolayer, well-defined brush border on the apical surface and intercellular junctions.
- Assessing transport in both directions (apical to basolateral (A-B) and basolateral to apical (B-A)) across the cell monolayer enables an efflux ratio to be determined which provides an indicator as to whether a compound undergoes active efflux.
- The P-glycoprotein (P-gp) inhibitor, verapamil, can be included to identify whether active transport is mediated by P-gp.



‘Studying the permeability of compounds across a Caco-2 cell monolayer is an established *in vitro* model to screen for oral absorption and to evaluate the mechanism of transport. Using LC-MS/MS for the analysis of samples derived from Caco-2 cell studies allows the rapid and accurate determination of drug transport across the Caco-2 cell monolayer.’

<sup>1</sup>Wang Z, Hop C.E., Leung K.H. and Pang J. (2000) *J Mass Spectrom* **35** (1); 71-6

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#### Protocol

#### Caco-2 permeability assay protocol

## Caco-2 permeability assay protocol

<b>Test Compound Concentration</b>	10 $\mu$ M
<b>Passage Number</b>	40 – 60
<b>Period of Cell Culture</b>	20 days
<b>Number of Replicates</b>	2
<b>Incubation time</b>	2 hours
<b>Temperature</b>	37°C
<b>Compound Requirements</b>	100 $\mu$ L of 10 mM DMSO solution
<b>Integrity Marker</b>	Lucifer Yellow
<b>Control Compounds</b>	Atenolol, propranolol and talinolol
<b>Analysis Method</b>	LC-MS/MS quantification
<b>Data Delivery</b>	$P_{app}$ Efflux ratio Experimental recovery



Cyprotex's Caco-2 assay is performed in a 96-well format providing a cost-effective and highly reproducible method of assessing the permeation potential of test compounds.

### Prediction of Human Intestinal Absorption

Cyprotex's Caco-2 Permeability data can be used in conjunction with [Cyprotex's Turbidimetric Solubility](#) data to predict dose dependent human intestinal absorption. Please refer to our [Human Intestinal Absorption Model](#) section for further details.

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### Data

## Data from Cyprotex's Caco-2 Permeability assay

For the validation, a set of compounds were screened through Cyprotex's Caco-2 Permeability assay over 3 separate experiments. Data generated were reproducible over a range of permeabilities.

The bidirectional assay is able to correctly distinguish between those compounds which are reported to undergo active efflux and those which are not.

Poorly permeable compounds

1

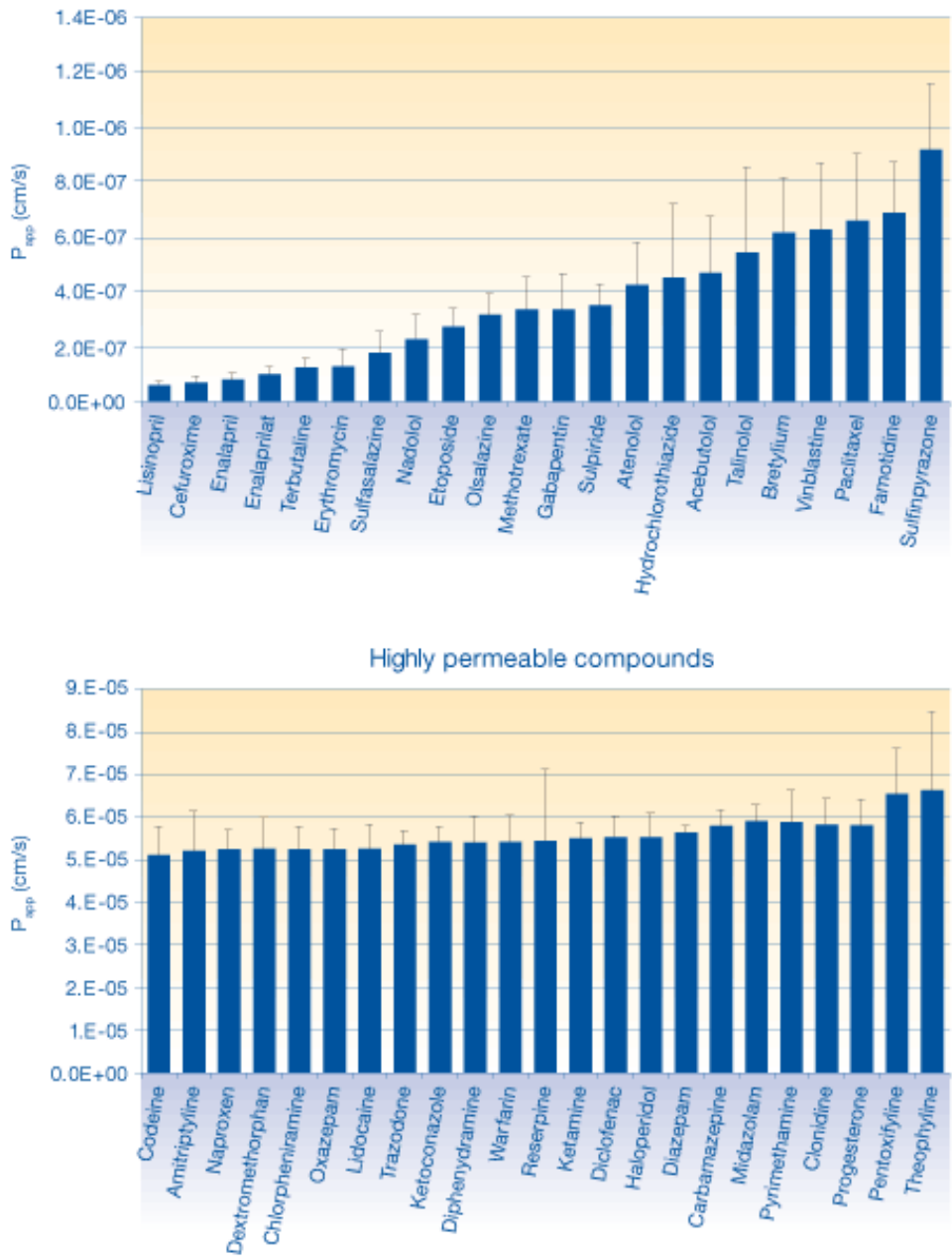


Figure 1

The graphs illustrate the consistency of Cyprotex's Caco-2 Permeability data over 3 separate experiments for the apical to basolateral assay.

These data illustrate the high level of reproducibility provided by this assay for a set of compounds with a range of permeabilities.

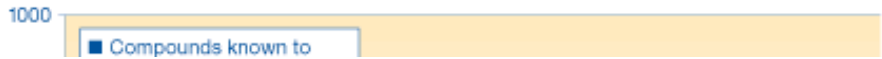
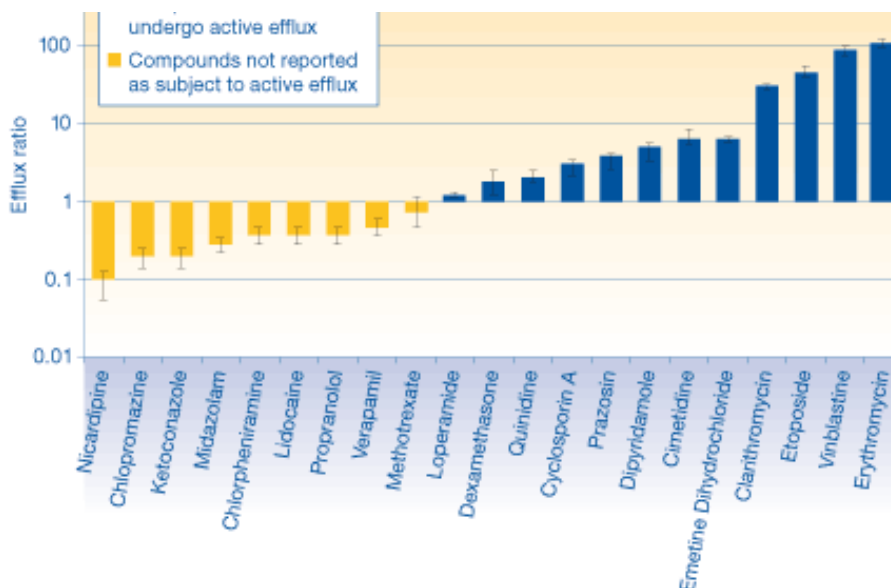


Figure 2



Graph illustrates the efflux ratio of a set of 21 compounds generated by Cyprotex's Caco-2 Permeability assay.

Cyprotex's bi-directional Caco-2 Permeability assay can identify and quantify levels of active efflux. Screening compounds in both the A to B and B to A direction provides a ratio of B-A/A-B (efflux ratio). When a compound has an efflux ratio of greater than 2, it suggests that the compound may be subject to active efflux.

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## Q&A

### Questions and answers on Caco-2 permeability

**Please provide an overview of Cyprotex's Caco-2 Permeability assay.**

Caco-2 cells are widely used as an *in vitro* model for predicting human drug absorption. The Caco-2 cell line is derived from a human colorectal carcinoma, and when cultured, the cells spontaneously differentiate into monolayers of polarized enterocytes.

The cells are seeded on Millipore Millicell plates and form a confluent monolayer over 20 days prior to the experiment. On day 20, the test compound is added to the apical side of the membrane and the transport of the compound across the monolayer is monitored over a 2 hour time period. To study drug efflux, it is also necessary to investigate transport of the compound from the basolateral compartment to the apical compartment.

The permeability coefficient ( $P_{app}$ ) is calculated from the following equation:

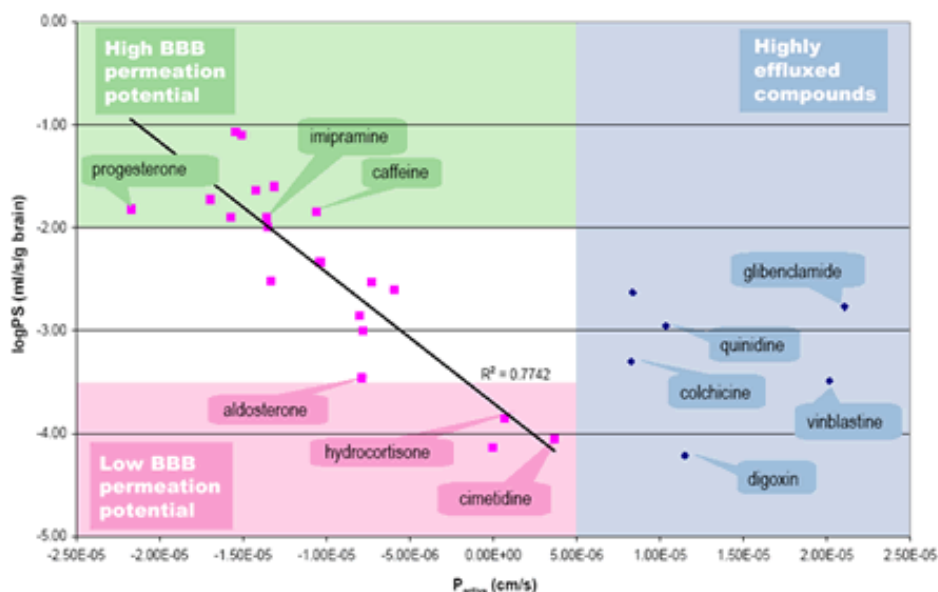
$$P_{app} = \left( \frac{dQ/dt}{C_0 \times A} \right)$$

Where  $dQ/dt$  is the rate of permeation of the drug across the cells,  $C_0$  is the donor compartment concentration at time zero and  $A$  is the area of the cell monolayer.  $C_0$  is obtained from analysis of the dosing solution at the start experiment.

## How do I interpret the data from the Caco-2 permeability assay?

There are several ways in which the data can be used. Firstly, the compounds can be ranked in terms of their Caco-2  $P_{app}$  values. Two reference compounds, atenolol (paracellular transport) and propranolol (passive transcellular transport) are screened alongside the test compounds. Atenolol and propranolol have known human absorption of 50% and 90% respectively<sup>2,3</sup>, and can be used as markers for ranking the test compounds. Secondly, the data can be used in conjunction with other *in vitro* parameters to predict the oral pharmacokinetics of a compound *in vivo* using the simulation software Cloe PK. Thirdly, the Caco-2 data can be used to predict blood brain barrier (BBB) permeability (Figure 3).

**Figure 3:** Correlation between Caco-2 derived  $P_{active}$  ( $(P_{app}^{B-A} - P_{app}^{A-B})/2$ ) and *in situ* BBB permeation rate, logPS (permeability-surface area product). Drugs known to permeate the BBB (caffeine, imipramine, progesterone) have logPS > -2.0 (green shaded area), whilst drugs known to not cross the BBB (aldosterone, hydrocortisone, cimetidine) have a logPS ≤ -3.5 (pink shaded area)

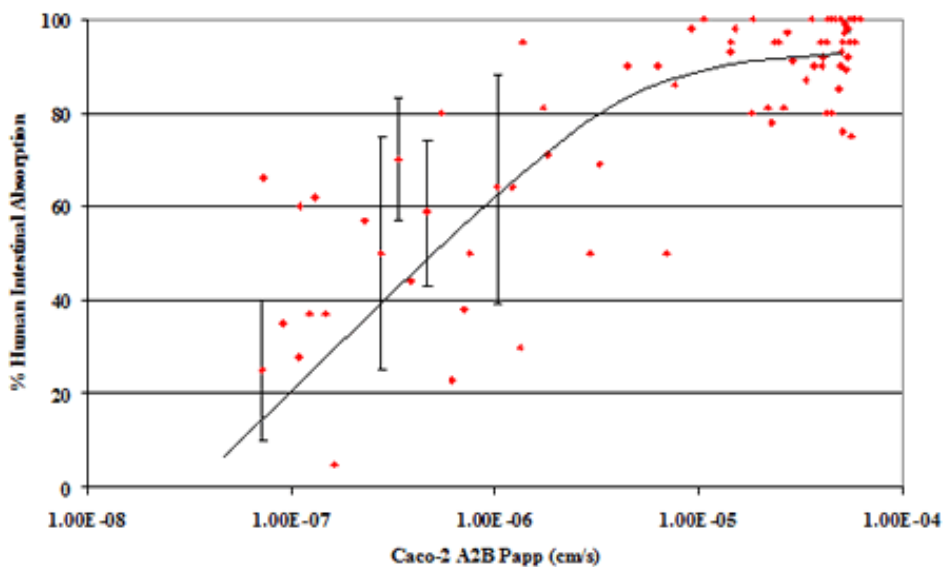


A regression line is shown which shows a clear correlation between  $P_{active}$  and logPS for compounds which are not subject to significant levels of efflux activity in the Caco-2 cells i.e., for compounds with a  $P_{active}$  of  $< 5 \times 10^{-6}$  cm/s. Using this correlation, Cyprotex's Caco-2 assay can be used to evaluate BBB potential for compounds with  $P_{active} < 5 \times 10^{-6}$  cm/s.

## What is the relationship between Caco-2 permeability and human intestinal absorption?

The relationship between Caco-2 permeability (using pH7.4 HBSS buffers in both the apical and basolateral compartments) and human intestinal absorption is displayed in Figure 4. This correlation is typical of those observed in the literature for these two parameters<sup>4</sup>. It is important to note that this plot is

influenced by the accuracy of the intestinal absorption data. The intestinal absorption values used in this plot are taken from Zhao *et al.* 2001<sup>2</sup>. In this paper the human intestinal absorption has been extracted from various sources and varies considerably in quality. Several compounds are also known to exhibit dose-dependent absorption (shown by error bars on the graph).



**Figure 4.** Relationship between Caco-2 permeability and % human intestinal absorption.

### What are the differences between the PAMPA and the Caco-2 permeability assay?

Caco-2 permeability screen is considered to be more representative of human absorption *in vivo* than PAMPA (parallel artificial membrane permeability assay). PAMPA solely provides a measure of passive diffusion whereas the Caco-2 model provides better prediction of the human absorption for compounds which display active uptake or efflux or pass through the membrane via the paracellular route. The information from both assays used in conjunction can be valuable in identifying the root cause for poor absorption.

### How do I measure drug efflux?

A bi-directional Caco-2 permeability assay is performed where the transport of the compound is measured in the apical to basolateral direction as well as the basolateral to apical direction. The result is typically reported as an efflux ratio i.e.  $P_{app}(B-A)/P_{app}(A-B)$ . If the efflux ratio is greater than two then this indicates drug efflux is occurring. Talinolol, a known P-gp substrate, is screened as a control compound to confirm that the cells are expressing functional efflux proteins. To identify if P-gp is responsible for the efflux of the test compound, verapamil, a P-gp inhibitor, can be included in the test compound incubation.

The efflux ratio should decrease in the presence of verapamil if the compound is a P-gp substrate.

## How do you know if the cells have formed a confluent monolayer?

Transepithelial electrical resistance (TEER) measurement is used to determine tight-junction formation between cells. Only wells with TEER values of at least 0.5Kohm are used in experiments. In addition, lucifer yellow, a membrane integrity marker, is co-incubated with the test compound at the start of the experiment. If the  $P_{app}$  of the lucifer yellow exceeds  $1 \times 10^{-6}$  cm/s then it is assumed that the formation of the cell monolayer has been unsuccessful and the compound is re-screened. If the lucifer yellow  $P_{app}$  continually fails for the same compound then it is assumed that the compound exhibits cytotoxic effects against the Caco-2 cells or that the compound itself is fluorescent.

## How and why is the % recovery calculated?

$$\% \text{Recovery} = \frac{\text{Total compound in donor and receiver at end of experiment (nmol)}}{\text{Initial compound present (nmol)}} \times 100$$

The % recovery can be useful in interpreting the Caco-2 data. If the recovery is very low, this may indicate problems with poor solubility, binding of the compound to the plate, metabolism by the Caco-2 cells or accumulation of the compound in the cell monolayer.

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## References

- <sup>1</sup> Wang Z *et al.* (2000) *J Mass Spectrom.* **35** (1); 71-6
- <sup>2</sup> Zhao YH *et al.* (2001) *J Pharmaceut Sci* **90**; 749-784
- <sup>3</sup> Yazdanian M *et al.* (1998) *Pharmaceut Res* **15**; 1490-1494
- <sup>4</sup> Kansy M *et al.* (2001) *Pharmacokinetic optimisation in drug research Ed. Testa et al*; 447-464

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