DB-ALM Protocol n° 142 : Permeability Assay on Caco-2 Cells

Intestinal Absorption, Mechanistic Studies, Systemic Toxicity

This protocol can be used to determine the permeability of test compounds across an *in-vitro* model of the human intestine: Caco-2 cells. The permeability is expressed as apparent permeability coefficient (P_{app}). *(Method evaluated in the EU Integrated Project "ACuteTox")*

Objective & Application

TYPE OF TESTING	:	Screening, Part of a testing strategy
LEVEL OF ASSESSMENT	:	Safety evaluation
PURPOSE OF TESTING	:	Mechanistic studies, Drug evaluation studies

Context of Use:

This protocol can be used for regulatory and non-regulatory purpose to determine the permeability of test compounds across an *in vitro* model of the human intestine. FDA's Guidance for Industry considers the data on drug absorption, obtained from the *in vitro* epithelial cell cultures for highly-permeable substances, as sufficient to justify a waiver of the *in vivo* bioavailability study (FDA, 2000).

Permeability Assay is not a direct test of toxicity. However, the biological endpoint measured here provides a key input parameter for physiologically-based toxicokinetic modeling (PBTK). PBTK in turn is regarded as an indispensable tool in the risk assessment of chemical substances by providing quantitative information about their penetration and their fate within a body (Adler et al., 2011; Coecke et al., 2013). This approach gains particular importance in the regulatory areas where the information on acute toxicity potential is necessary but the *in vivo* experiments are either banned or considered only as a last resort:

- cosmetic ingredients and preparations (EC regulation No. 1223/2009; EU, 2009),
- chemicals (EC regulation No. 1907/2006; EU, 2006),
- biocide preparations (EC regulation No. 528/2012; EU, 2012),
- plant protection products (EC Regulation No. 1107/2009; EU, 2009)

The **Permeability Assay** is one of the 11 test methods that were selected from 53 candidates for building blocks of of a non-animal testing strategy for predicting human acute oral toxicity, which was the main aim of the EU Integrated project AcuteTox (**www.acutetox.eu**). In the second stage of the project, the reliability of the method was evaluated against a new blind-coded set of 32 chemicals (Prieto et al., 2013).

Applicability Domain:

Caco-2 cell permeability is one of the most frequently used assays to predict human oral absorption of drug candidates and in drug pre-clinical evaluation in the pharmaceutical industry. Numerous publications discuss its use, in particular: the retrospective analysis from 1991 to present by Artursson P. and Karlsson J. (1991) and Pelkonen *et al.* (2001). In the context of the ACuteTox project, the chemicals which were successfully tested with this protocol cover several uses: industrial chemicals, pharmaceuticals, agrochemicals, food additives and an environmental toxin (Prieto et al., 2013).

Résumé

Human colon carcinoma Caco-2 cells are widely used as model for the human intestinal epithelium. Since a correlation was shown between oral drug absorption and apparent drug permeability coefficients for transport across Caco-2 monolayers, this cell model became very popular for *in vitro* prediction of drug uptake (Artursson and Karlsson 1991, Tavelin *et al*. 2002, Artursson *et al.,* 1996).

Caco-2 cells are used as a rate-limiting barrier model of the human intestinal epithelium for uptake of xenobiotics after oral ingestion. Many reviews have been written about the usefulness and applications of Caco-2 cell monolayers in determining intestinal drug absorption (e.g. Tavelin *et al.,* 2002; Shah *et al.,* 2006).

Experimental Description

Endpoint and Endpoint Measurement:

PERMEABILITY COEFFICIENT: The assay measures the permeability from the apical to the basolateral side and vice versa, ideally at lowest concentration limited by detection and up to the highest non-toxic concentration

Endpoint Value:

P_{app} - the value of the apparent permeability coefficient expressed as (cm x s^{-1})

Experimental System(s):

Caco-2 cell line (human)

Basic Procedure

The cell culture part of the Protocol is subdivided in two parts:

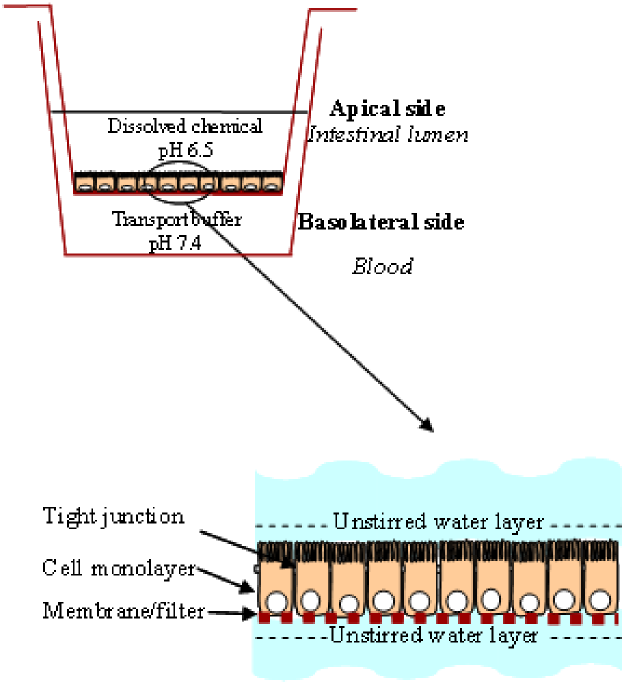
1. Maintenance of cells in flasks (routine culture/ subculture) and
2. Seeding on Transwell-filters and maintenance of cells on filter supports

Routine culture is required for maintenance and to obtain sufficient amounts of cells for seeding onto filter supports which are used in the actual measurement. The routine culture in flasks has to be maintained for approximately 1 week before the seeding onto filter supports.

The transport experiment is preceded by:

1. *An evaluation of the effect of the test compounds on the Caco-2 monolayer integrity, referred further as "Toxicity test"*
   In this evaluation experiment, the test compound is applied together with a paracellular marker that permeates intact monolayers very slowly, and the permeability of the paracellular marker in presence of the test compound is compared to the one in absence of the test compound. Impaired integrity is reflected by an increase in permeability of the paracellular marker. If the test compound does not damage the cell-monolayer integrity, the transport experiment can be conducted up to the identified non-toxic concentration.
2. *Cell monolayer integrity control*
   Before the transport experiment is started, the trans-epithelial electrical resistance of the cell monolayer is measured as check of monolayer integrity.

The figure below illustrates one test chamber (longitudinal section) of the 12-well-plate including the filter-support covered with a cell-monolayer. The filter with the cell-monolayer divides the well into two sections (apical and basolateral), allowing for the dissolved compound to be applied on one side (donor), while the other side (receiver) at t=0 contains only buffer. The whole experimental device is well agitated during the experiment to reduce the unstirred water layers on top and below the filter. Samples are withdrawn at defined time points from the receiver side and the amount of compound which crossed the cell-monolayer per time interval is determined. The apparent permeability coefficient (P_{app}) is determined from the amount of compound transported per time unit.



Data Analysis/Prediction Model

The P_{app}-values are calculated from the concentration of the un-metabolised compounds after transport across the cell monolayers. If metabolism occurs, the transport of metabolites is not taken into account.

P_{app}-classification is based on correlation of Caco-2 permeability coefficients and in vivo "fraction absorbed" data for model compounds. Low (0-20%), intermediate (20-70%) and high (70-100%)

Test Compounds and Results Summary

Table I: Examples for permeability coefficients determined with Caco-2 monolayers (pH 7.4)
data from Bergström, C. et al. (2003), Stenberg, P. et al. (2001)

Substance	P_{app} [x 10^{-6} cm s^{-1}]	P_{app}-classification
amitriptyline	126	high
ethinyl estradiol	378	high
warfarin	59	high
amiloride	0.3	intermediate
lactulose	0.3	intermediate
raffinose	0.051	low

The results obtained within the ACuteTox project, have been submitted to an special issue of Toxicology in Vitro, dedicated to this project (Kinsner-Ovaskainen et al. 2013 and Prieto et al. 2013). From 32 selected chemicals only 18 produced results with this protocol, whereas the remaining 14 failed due to a lack of a suitable analytical method.

Modifications of the Method

In special case of substances with high adsorption to plastic, 4% BSA can be added to the basolateral recipient solution, to bind the lipophilic substances to protein instead of plastic and so to improve the mass balance (Neuhoff *et al.*, 2006). Prior to the endpoint measurement, BSA must be removed by precipitation in the presence of acetonitrile.

Discussion

Ethical issues

The test method is based on the commercially available Caco-2 cell line. The only animal-derived products used in this test are foetal calf serum and BSA.

Special equipment

A laboratory performing the test must have access to appropriate analytical equipment (HPLC and/or LC-MS instrument) and a device for Trans Epithelial Electric Resistance (TEER) Measurements.

Required Training and Expertise

Caco-2 cell permeability is a very well established and documented assay in the pharmaceutical industry. Nevertheless, it requires adequate experimental skills and expertise in the handling of the cell culture, and in the validation of analytical and computerized methods.

[^{14}C]-Mannitol is used in the course of the assay, and consequently the testing laboratory must adhere to the local regulations regarding the procurement, handling and disposal of radioactive materials.

Limitations and key attention points

Passage number

Cell lines may change characteristics when sub-cultured for a long time; use cells within a defined interval during one project, i.e. use 10-15 passages before thawing a new cell-vial.

Lipophilic properties of the test compound

Compounds with high clogD-values (>4.5) may pose problems in the determination of permeability since these compounds are often poorly soluble in aqueous solvents and might precipitate in the course of experiment. In addition such compounds often adsorb to plastics (i.e. all parts of the experimental device), which may change the donor concentration, resulting in a disappearance from the receiver solution and loss during sampling/analytics. One possible solution is an introduction of bovine serum albumin (BSA) into the basolateral compartment, resulting in :

1. an additional driving force during the transport (uptake direction) and
2. analytical challenges (Neuhoff et al. 2006).

Detection and quantification of a test compound

One limitation of this protocol is the availability of a reliable analytical method for quantification of a test compound. It must be capable of quantification of the concentrations typically occurring in this assay (the 1-1000nM range). Most versatile analytical methods are based on the LC-MS/MS, however some compounds are not compatible with such instruments.

In the course of the ACuteTox project, problems were encountered with regard to the lack of an analytical method for several non-pharmaceuticals, e.g.: Tween 20, sodium bicarbonate, potassium sulphate, potassium chloride, and dimethyldioctadecylammonium chloride (Prieto et al. 2013).

In order to detect a low-permeable compound and estimate P_{app} accurately, the analytical sensitivity needed may be below 1/1000th of the donor concentration.

For example: To detect a low-permeable compound (around 0.5 x 10^{-6} cm/s) at a donor concentration of 10 µM, an analytical method needs a limit of quantification below 10 nM. Otherwise it is not sensitive enough to determine the concentration of the test compound in the recipient compartment, needed to calculate mass balance and P_{app}.

If the detection limit is too high (insufficient), one may try to either:

1. increase the donor concentration (with a considerable risk to saturate transport proteins and metabolizing enzymes) or
2. increase the time intervals between the samplings (with the risk to loose the sink property in the receiver compartment and to under-determinate the P_{app}-values).

Furthermore, the donor concentration may be limited by the upper solubility range of the compound.

Ionisation state of test compound in experimental pH

The setup for the experiments simulates the pH-values in the upper small intestine, pH 6.5 in the apical compartment (luminal side) and pH 7.4 in the basolateral compartment (abluminal side) leading to a pH-gradient-system.

In this case, when P_{app}-values are determined for both directions, uptake (ab) and efflux (ba), a comparison between these values can give rise to “pseudoefflux” (basic substances) or “pseudoinflux” (acidic substances), which is not dependent on transporter-proteins in the cell membranes but on the different pH-values in the donor and receiver compartments. So, without the knowledge of the effect of ionisation of the compound in the different compartments, the efflux or influx ratios should not be interpreted directly as transport protein involvement (Neuhoff et al. 2003, Neuhoff et al. 2005).

Status

Known Laboratory Use:

Academia (e.g.: Uppsala University, Sweden;)

The assay is commonly used at most private contract research organisations who deal with pharmaceuticals.

Participation in Evaluation Studies:

The method was evaluated in the Phase I of ACuteTox, an EU integrated project with the aim to develop and assess a simple and robust *in vitro* testing strategy for prediction of human acute oral systemic toxicity.

Participation in Validation Studies:

After the Phase I of the ACuteTox project (in collaboration with EURL ECVAM) the Permeability Assay was among 11 methods selected from 53 candidate in vitro and in silico test methods. All methods were evaluated in terms of variability and preliminary predictive capacity to classify chemicals into the EU CLP acute oral toxicity categories (EC No. 1272/2008; EU, 2008; Kinsner-Ovaskainen et al., 2013).
The reliability of the Permeability Assay method was further evaluated during the Phase II of the project with a defined set of 32 selected chemicals (Prieto et al., 2013). It was considered as sound source of one of the key input values for the algorithms which estimate a correlation between the oral dose and the effective concentration of the test compound (Blaauboer and Garberg- in preparation)

Regulatory Acceptance:

Caco-2 Permeability assay is a well established method in the pharmaceutical industry 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 information it provides is compliant with the pre-clinical safety assessment described in the ICH guidelines M3(R2) and S7A (ICH, 2009 and 2000). Furthermore FDA considers the data on drug absorption, obtained from the *in vitro* epithelial cell cultures for highly-permeable substances, as sufficient to justify a waiver of the *in vivo* bioavailability study (Guidance for Industry; FDA, 2000).

Health and Safety Issues

General Precautions

When culturing the cells, gloves and laboratory coat must be worn by operators. Only sterile equipment must be used in cell handling. Discard all the materials utilised following the appropriate procedure for special biological waste. During handling of cryogenic vials in liquid nitrogen, a full-face mask and gloves must be worn. All solutions, glassware, pipettes, etc., shall be sterile and all procedures should be carried out under aseptic conditions and in the sterile environment of a laminar flow cabinet (biological hazard standard).

All uncharacterised and coded test compounds should be considered as potentially toxic and must be handled with maximum care.

Radioactive compounds must be handled according to radiation safety standards

Abbreviations and Definitions

ab: apical-to-basolateral

ACuteTox: EU FP6 integrated project for Optimisation and Prevalidation of an *In-Vitro* Test Strategy for Predicting Human Acute Toxicity

ba: basolateral-to-apical

BSA: bovine serum albumin

clogP: calculated log P (logP = partition coefficient between octanol and water)

clogD: calculated partition coefficient between octanol and water at defined pH

DMEM: Dulbecco´s modified Eagle´s medium high glucose

DMSO: dimethylsulfoxide

EC: European Commission

EDTA: ethylenediaminetetraacetic acid, disodium salt

EU: European Union

EURL ECVAM: European Union Reference Laboratory for the Validation of Alternative Methods (formerly referred to as ECVAM)

FDA: US Federal Drug Agency

FCS: foetal calf serum

FP6: EU-funded Framework Programme for Research and Technological Development, took place from 2002 until 2006.

HPLC: High-performance liquid chromatography

ICH: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use

LAF-bench: laminar air flow bench

LC-MS: liquid chromatography electrospray ionization mass spectrometry

NEAA: nonessential amino acids

P_{app}: apparent permeability coefficient - **Papp = dQ / dt * 1/(A*C0)**

PBS: phosphate buffered saline

Pgp: P-glycoprotein

PepT1: peptide transporter 1

PEST: penicillin-streptomycin solution

TEER: trans-epithelial electrical resistance

Last update: 27 March 2013

PROCEDURE DETAILS, 27 January 2012

Permeability Assay on Caco-2 Cells
DB-ALM Protocol n° 142

This test method has been evaluated in the FP6 integrated project ACuteTox, contract number 512051 (years 2005-2010).

An absolute prerequisite for the use of this protocol is the availability of a well established and reliable analytical method for the quantification of the test compound (not included in the protocol).

Contact Details

Prof. Per Artursson
Department of Pharmacy
Uppsala University
email: per.artursson@farmaci.uu.se

Dr. Carrie Tsoi
Department of Pharmacy
Uppsala University
email: carrie.tsoi@farmaci.uu.se

Dr. Richard Svensson
Department of Pharmacy
Uppsala University
email: richard.svensson@farmaci.uu.se

Materials and Preparations

Cell or Test System

Caco-2 cells, ATCC. HTB-37™

Equipment

Fixed Equipment

a. Laminar air flow bench with aspirating device and pipetting aid (e.g. Pipette boy, Integra Biosciences) (+ beaker with Jodopax for aseptical decantation)
b. Cell counter (e.g. NucleoCounter, ChemoMetec A/S Allrød /Denmark)
c. Inverted microscope (10x objective and 10x ocular)
d. Benchtop-centrifuge for 50 mL Falcon-tubes (e.g. Centaur 2, MSE Measuring & Scientific Equipment Ltd., Crawley, England)
e. Freezers, -20°C, -80°C
f. Fridge
g. Nalgene-box for freezing, cryo 1°C, cat-no. 5100-0001
h. Water-bath
i. Tweezers
j. Volume adjustable pipettes (up to 1 mL and up to 200 mL)
k. Incubator (37°C) with CO_2-connection (10%) and humidity (95%) and calibrated orbital shaker (IKA-Schüttler MTS4)
l. Heating plate during sampling (37°C)
m. pH-meter with electrodes and calibration solutions
n. Liquid handling pipettes with disposable tips in adequate adjustable volumes (e.g. 0.1-1 mL, 20-200 µL, 10-100 µL, 5-40 µL; suppliers Gilson, Thermo or Eppendorf)
o. Positive-displacement pipette with disposable tips and capillary pistons for pipetting the radioactive stock-solutions (e.g. Microman 25, Gilson)
p. TER-measurement device: Endohm tissue resistance measurement chamber (WPI, Sarasota, Florida/USA) with Millicell-ERS-voltmeter (Millipore, Bedford, MA/USA) or with Evohm epithelial voltmeter (WPI, Sarasota, Florida/USA)
q. Balances in adequate ranges (including one micro-balance, e.g. Sartorius-micro)
r. Magnetic stirrer
s. Scintillation counter + scintillation vials
t. Working bench
u. Vortex
v. Volumetric flasks

w. Glass vials with caps for stock solutions
x. For analytics:
 - plate-reader UV-Vis (e.g. Safire [2], Tecan/Switzerland)
 - **HPLC with autosampler and UV-detection** (e.g. Bischoff/Germany with Midas autosampler Spark/The Netherlands)
 - **HPLC-MS with autosampler** (e.g. Finnigan TSQQuantum Discovery with Finnigan surveyor MS-pump and Finnigan surveyor autosampler /USA)
 - HPLC-vials (+ caps) and 96-well-deep-well plates
 - HPLC-columns

Consumables

a. Disposable serological pipettes: 1 mL, 5 mL, 10 mL and 25 mL Stripette® and pipetting help (e.g. Corning costar)
b. 12-well cell culture clusters: Corning costar
c. Cell culture flasks: 25 cm^2, 75 cm^2 and 162 cm^2 (e.g.Corning costar)
d. Filters: Corning costar Transwell® permeable supports, polycarbonate membrane pore size 0.4 µm, diameter 12 mm (12-well-plates,cat.no. 3401), or 24 mm (6-well-plates, cat no. 3412) or corresponding product from Millipore
e. Nucleo-casettes for cell counter, ChemoMetec A/S Allrød /Denmark
f. Falcon-tubes (50 mL and 15 mL)
g. Eppendorf-tubes
h. Cryovials
i. Isopropanol
j. Jodopax vet (5% iodine and 15% acetic acid stock, e.g. Pharmacia AB, Helsingborg).
 Before use dilute 1+1 with water and take 2 mL into 1 L-beaker

Media, Reagents, Sera, others

a. Dulbecco´s modified Eagle´s medium (DMEM) high glucose (4500 mg/L glucose) with L-glutamine (without pyruvate) (e.g. Gibco / Invitrogen, cat.no. #. 41965-039)
b. Foetal calf serum (FCS); not heat inactivated. Store at –70 (long term) /-20°C (short term), thaw in fridge, aliquot after thawing and store aliquots at -20°C.

 NOTE: The results of transport experiments may depend on the batch of serum used. Therefore, before purchasing a large batch of serum, it is recommended to test 6-7 different sera for cell growth, integrity and active trans-epithelial transport. Furthermore, all new FCS batches need to be tested before performing the experiments.
 Briefly, the test procedure is done as follows: cells are cultured for two weeks and during this period the old serum is replaced by the new one using a stepwise procedure. The cells are then seeded on filters and cultivated for 3 weeks before the transport experiments for serum evaluation are done. The transport tests include mannitol (integrity marker), glycyl-sarcosine (active transported by PepT1) and a Pgp-substrate.

c. Minimal essential medium nonessential amino acids (NEAA), 100x, without L-glutamine (e.g. Gibco / Invitrogen # 11140-035, 100 mL), store at 4°C
d. PEST solution
 penicillin 10 000 u/mL - streptomycin 10 000 µg/mL – (100x) (e.g. Gibco / Invitrogen #15140-122)– store at -20°C; aliquot after thawing and store aliquots at -20°C
e. Trypsin solution 2.5% (10x)
 (e.g. Gibco /Invitrogen #15090046, 100 mL; or Sigma # T4549, 100 mL) – store at -20°C; aliquot after thawing and store aliquots at -20°C
f. EDTA 2% (sodium salt) solution; (e.g ICN Biomedicals # 2820549) – store at room temperature
g. Phosphate-buffered saline (PBS), Dulbecco´s, without Ca, Mg, 1x (e.g. Gibco /Invitrogen #14190094, 500 mL)
h. DMSO > 99.9% A.C.S reagent (e.g. Sigma # 472301), filter sterilised across hydrophobic fluorofore (PTFE) membrane for fine particle removal from organic solvents 0.2 µm Millex FG (Millipore), stored at -20°C in sterile vials
i. Ice
j. Hanks balanced salt solution (HBSS) without phenol red, powder (e.g. Sigma, 10 packages for 1L each, # H1387)
k. HEPES Sigma Biotechnology Performance certified (e.g. #H-4034)
l. 2-Morpholinoethanesulfonic acid (MES) Sigma, cell culture grade, or Fluka, BiochemikaUltra for molecular biology, (e.g. #69889 (monohydrate))
m. $NaHCO_3$
n. MilliQ-water
o. HCl
p. NaOH

q. DMSO 99.5% GC-grade (e.g. Sigma #D5879)
r. Acetonitrile, (e.g. LiChroSolv Merck (VWR) #1.06007)
s. Methanol, (e.g. LiChro gradient Merck (VWR) # 1.14291)
t. Formic acid for HPLC-MS (e.g.Fluka #94318)
u. Ammonium acetate(e.g. Fluka #17836)
v. **[^{14}C]-Mannitol** (e.g. NEN Life Sciences)

Preparations

Media and Endpoint Assay Solutions

Work in LAF-bench made aseptic with ethanol before and after work; use only sterile material.

DMEM-medium, complete

Add additives into the DMEM-bottle:

1. DMEM-medium for maintenance of the cells in flasks (**DMEM complete**)

   500 mL DMEM
   50 mL FCS
   5 mL NEAA

2. DMEM-medium for maintenance of the cells on filters (**DMEM-PEST**)

   500 mL DMEM
   50 mL FCS
   5 mL NEAA
   5 mL PEST

Trypsination solution (0,25% trypsin/0,2 %EDTA)

40 mL PBS
5 mL EDTA
5 mL trypsin

mix in 25 cm^2 (50 mL) cell culture flask; store at 4°C

HBSS/Hepes pH 7.4

For preparation of 1 L:

HBSS powder - 1 package
$NaHCO_3$ - 0.35 g
HEPES (final concentration 25 mM) 5.95 g

Dissolve components in MQ-water (about 950 mL). Adjust pH to 7.33 – 7.38 with NaOH. Adjust volume to 1 L (volumetric flask). Filter sterilise through 0.22 µm pore filter and store in autoclaved bottles, in the dark at room temperature. Check pH in before the experiment and adjust, if necessary, to 7.4.

In special cases (working with substances with high adsorption to plastic) it may be necessary to include 4% BSA (w/v; BSA fraction V) into the HBSS; store at 4°C.

HBSS/MES pH 6.5

For preparation of 1 L:

HBSS powder - 1 package
$NaHCO_3$ - 0.35 g
MES monohydrate (final concentration 10 mM) 2.13 g
Dissolve components in MQ-water (about 950 mL).

Adjust pH to about 6.4 with NaOH.
Adjust volume to 1 L (volumetric flask).

Filter sterilise through 0.22 µm pore filter and store in autoclaved bottles, at room temperature in the dark.

Check pH in before the experiment and adjust, if necessary, to 6.5.

Test Compounds

Solubility Test

1st Target: 2 mM solution in HBSS-MES, i.e. twice the highest concentration applied in the toxicity test.

To Do: Weigh appropriate amount of test compound, add HBSS-MES to reach 2 mM concentration of the compound, vortex for 10 s.

Check visually:

- If the compound is not solubilised, warm it in a water bath to 37°C and check again.
- If the compound is still not solubilised, test with 1 mM (addition of appropriate amount HBSS-MES possible)
- If the compound is still not solublised, test 0.2 mM
- If not buffer-soluble at least 0.1 mM go to 2nd Target

2nd Target: 100 mM in DMSO, i.e. 100 times the highest concentration applied in the toxicity test; DMSO-stocks are prepared in glass-vials.

To Do: The same procedure as for 1st Target, but:

- After dissolution in DMSO, dilute the DMSO-stock 100-times with HBSS-MES and check if the compound is still dissolved after dilution in the water phase; use glass-vials.
- If precipitation is observed, a 10-times lower concentration in the DMSO stock has to be tested and so on.

Check of Solution Concentration

P_{app}-values are calculated by relating concentrations on the receiver side to the donor side Smaller errors are cancelled out by the calculation procedure.

For compounds with high clogP, plastic adsorption problems can be detected by comparing HPLC (or HPLC-MS) signals of the compound-HBSS-1%DMSO solution done direct before analysis into the glass autosampler vial and the signal of the solution after application (1 h) onto the 12-well plate.

Solubility can be checked by comparing the HPLC (or HPLC-MS) signals of the compound diluted with either HBSS/1%DMSO or 50% acetonitrile / 50% water.

pH of Test compound Solutions

The pH is measured in the HBSS-solution directly after test for solubility. If titration is needed, the amount and composition of the acid/base added must be recorded.

Working Concentrations of Test compound

For toxicity tests: 1 mM, 0.1 mM, 0.01 mM (if applicable, i.e. if the compound is easily dissolved).

For transport experiment: Only concentrations that do not impair the monolayer-integrity can be used. Ideally, the lowest concentration limited by detection should be used. Higher concentration increases the risk of saturation of transport proteins. Recommended donor-concentrations: 1 mM (with UV-based analysis) or 0.01 mM (with HPLC-MS analysis). Lower concentration must be used if dissolution problems or toxicity are observed.

Positive Control(s)

Mannitol (^{14}C) is used as a cellular integrity marker together with the test compound. At this moment no standard reference for P_{app} determination has been established.

Negative Control(s)

Not applicable.

Method

Routine Culture Procedure

Routine incubation conditions: 37°C, 10% CO_2, 95 % humidified atmosphere. The medium is changed every second day, i.e. Monday (splitting), Wednesday, and Friday. Work in LAF-bench made aseptic with ethanol before and after work. Use sterile materials only.

Thawing Cells

This procedure will be done preferably on a Thursday (notice that medium has to be changed on the following day). On the following Monday, a sufficient confluent cell-culture will be available for trypsination and propagation into the next culture flask.

a. Prepare the cell culture medium supplemented with FCS (DMEM-medium, complete) and pre-warm about 20 mL, keeping the rest fridge-cold.
b. Remove the vial from the liquid nitrogen container. Use gloves and head screen-protection or plastic glasses. Place the vial on ice immediately for transport to water-bath and LAF-bench.
c. In the clean room, transfer the vial to a water bath that has been warmed to 37°C.
d. At the moment when the ice is reduced to a small sphere (only a nucleus looks still yellowish and the surrounding

medium has turned red-pink), immediately remove the vial from the water bath and swirl the cell suspension until the residual ice has melted.

e. In the LAF-bench transfer the cell suspension to a centrifuge tube and slowly (drop-by-drop) add 5 ml fridge-cold culture medium. Mix the cell suspension carefully while adding the medium.
f. Spin the cells down at 1200 rpm for 5 min. Aspirate the supernatant.
g. Re-suspend the cells carefully in fresh pre-warmed cell culture medium (15 mL) and transfer to a culture flask (25cm ^{2} flask).
h. Place the flask in the CO_2 incubator with the proper CO_2 content and incubate overnight. On the next day verify if the majority of the cells had attached to the flask surface and remove the medium with dead cells and cell debris. Replace it with fresh cell culture medium.

Cell Counting

Perform according to the NucleoCounter instructions.

Trypsination, seeding during passaging and maintenance of the cells in flasks

Trypsination:

When the cell monolayer is about 90 % confluent:

a. Remove the medium from the cells (aseptical decantation into a beaker with 2 mL diluted iodopax solution);
b. Rinse with PBS (15 mL/75 cm ^{2}-flask, 30 mL/162 cm ^{2}-flask);
c. Remove PBS and add trypsin/EDTA solution (5 mL/75 cm ^{2}-flask, 10 mL/162 cm ^{2}-flask);
d. Rinse the cells and pour off the majority of the trypsin solution, so that only about 500 mL – 1mL remain in the flask; the remaining trypsin solution shall wet the whole cell-surface;
e. Close the flask and incubate at 37°C for 6 – 15 min (checking the progress in between);
f. Help to release the cells from the plastic surface by carefully knocking on the flask;
g. As soon as all cells are released, stop the trypsination by adding complete DMEM (8 – 10 mL) followed by resuspension of the cells by pipetting up and down several times;
h. After transfer to a Falcon tube (50 mL) containing 10 mL complete DMEM, note the volume of the cell suspension;
i. Suspended the cells by carefully pipetting up and down several times so that all aggregates are dipersed and take an aliquote for cell counting
j. Spin the cells down (5 min at 1500 rpm, 16 cm swing-bucket rotor, i.e. ca. 400 g).
k. Remove the supernatant, resuspend the cell pellet carefully in complete DMEM to a final concentration 1 x 10 ^{6} cells/mL.
l. Count the cells from the aliquot using the Nucleo-casettes.

Passaging:

The aim is to get the cells to reach about 90% confluency after a week. Seed 0.3– 1.2 x 10 ^{6} cells/75 cm ^{2} -flask in 15 mL complete DMEM; 2 x 10 ^{6} cells/162 cm ^{2}-flask in 30 mL complete DMEM. Write down cell passage number in your experimental recods.

Volumes after medium change:

25 ml/75 cm ^{2}-flask, 50 mL/162 cm ^{2}-flask

Freezing Cells

a. Prepare the freezing vials; Prepare 6 (or multiples of 6) vials per freezing block. Use the 1ml cryovials with the silicon band around the lock. Mark them with the ethanol-proof permanent marker pen with:
    - name of the cell line and/or clone
    - passage number upon freezing
    - date of freezing
b. Pre-cool the marked vials at -20°C
c. Prepare the Nalgene-freeze box (cryo 1°C): add isopropanol up to the mark and place box in fridge
d. Prepare 2x stock solution of DMSO (20%) in the freezing medium by mixing 2 ml DMSO and 8 ml FCS-containing medium. Place this solution on ice.
e. Prepare the cells using the regular trypsination protocol described in the previous section. Wash with PBS, trypsinize, inactivate trypsin with FCS-containing medium, count the cells, spin the cell suspension down, discard supernatant. Target concentration for resuspension: 8x10 ^{6} cells/mL (i.e. double the concentration which will be frozen). Re-suspend in a half of the final volume with the culture medium. Store the cell suspension on ice.
f. Add the other half of the final volume to the cell suspension by adding pre-cooled 20%-DMSO solution drop by drop with gentle swirling (do not work too slowly). In this step DMSO is dilluted to a final concentration of 10%

and final volume of the cell suspension is reached (giving 4x10 ^{6} cells/mL).

g. After final re-suspending of the cells, quickly aliquot the suspension into freezing vials (1 mL each), tighten the lock on vials (but not over tight), and place the vials into the pre-cooled Nalgene-freezing-box at -84°C for at least 4 h or overnight.
h. Afterwards, quickly remove the vials from –84°C and place them into liquid nitrogen container (Use gloves and head screen-protection or plastic glasses). Be careful to choose the correct container and properly marked storage block.
i. Make a record in the freezer log with the following information:
    - name of the cell line and/or clone
    - culturing medium (and/or freezing medium if it is different from culturing medium)
    - passage number upon freezing
    - cell number per vial
    - date of freezing
    - number and position of stored vials
    - confirmation of mycoplasm-absence
j. Check if the freezing process was successful by thawing one vial later. Make the note into the log that one vial was taken out and when. At the same time check the cells for mycoplasma contamination.

Seeding on Transwell-filters and maintenance of cells on filter supports

Cells are trypsinated as described above. Before seeding, the cells are resuspended in the complete DMEM without PEST.

Seeding:

- Resuspend the cells in complete DMEM (0.66 x 10 ^{6} cells/mL)
- The filters (12 mm diameter) are pre-wetted with about 0.1 mL medium before seeding the cells (0.5 mL for 24 mm-filters); use ethanol-washed tweezers to place all filters in the same direction in the 12-well-cluster (open part of the ring at 15-30 min -position)
- Seeding density: 2 to 4.4 x 10 ^{5} cells/cm ^{2} (225 000 to 500 000 cells/12 mm diameter filter), (e.g. during the EU FP6 integrated project ACuteTox: 2.9 x 10 ^{5} cells/cm ^{2} (330 000 cells/12 mm diameter filter))
- The basolateral chamber is filled with DMEM-PEST (1.5 mL for 12-well-plates; 2.5 mL for 6-well plates).

Medium change (DMEM-PEST):

Medium change should be performed every other day except over the weeked. (monday, wednesday and friday).

The medium is first aspirated from the basolateral side of all wells, then carefully and slowly from the apical side (of all filters in a plate); add first medium in the apical compartment and then in the basolateral one (DO NOT touch the filter surface!).

- 12-well plates: 1.5 mL basolateral, 0.5 mL apical (but 0.6 mL on Fridays)
- 6-well plates: 2.5 mL basolateral, 2.5 mL apical

Test Material Exposure Procedures

The cells are seeded on filter supports 3 weeks before the experiment and maintained as described in the **Routine Culture Procedure**.

The transport experiment is performed 21-29 days after seeding (standard: at day 24 +/- 2)

1. The influence of the test compound on cell-monolayer integrity (“Toxicity test”)

[^{14}C]-mannitol is used as a paracellular marker and the monolayer integrity is evaluated by comparing the fluxes of [^{14}C]-mannitol in the presence and absence of a compound to be tested (sampling times 30, 60, and 120 min). The transport experiment to determine the mannitol-flux is performed as described below (2. Transport experiment).

If the compound to be tested is dissolved in DMSO, the control experiments in absence of the compound are also performed with DMSO (1% in both chambers as for the experiment in presence of the compound).

2. Transport experiment

NOTE: During the transport experiments, the filters with the cell monolayers have to be well agitated to overcome the "unstirred water-layer" effects (limiting permeation); otherwise P_{app}-values can be underdetermined.

- Use Caco-2 monolayers grown on filter inserts after 21-29 days of culture (standard 24+/-2 days) and change the culture medium 12 – 24 h before the experiment
- The experiments are done at least in triplicate
- Incubations: at 37°C in humidified atmosphere (**but without CO_2**)
- All solutions used are pre-warmed at 37°C
- *Co-solvent*: add only if the compound does not dissolve in HBSS. In this case, DMSO (or ethanol, if the compound does not dissolve in DMSO) is used. The DMSO concentration during the experiment is 1% (v/v) and it is included in both the apical and basolateral compartments as well as in the replacement HBSS-solution.
- *pH:* pH 6.5 (HBSS-MES) in the apical and pH 7.4 in the basolateral chambers (HBSS-Hepes). The experimental set-up is chosen to simulate the pH-values in the upper small intestine, with a lower pH in the bowel than in the blood, set experimental to pH 6.5 in the apical compartment ("intestine") and pH 7.4 in the basolateral compartment ("blood"), which is physiologically human intestine-like.

2.1 Wash

Monolayers grown on filter inserts are washed carefully to remove residual culture medium (medium shoudl be decanted, not aspirated): the filter inserts with the cell monolayers are transferred into new multi well plates containing 1.5 mL of buffered HBSS/well (12-well plate) and buffered HBSS is added to the apical side (0.5 mL/12 mm-filter).

The filter inserts are incubated with shaking (100 rpm) for 15-20 min.

2.2 Experiment

- The compound of interest is dissolved in buffered HBSS at the desired concentration (donor solution). The pH of the donor-solution is checked and, if necessary, adjusted to 6.5.
- A ***replacement experiment*** (i.e. a sample is withdrawn from the receiver chamber at a specific time-point and the withdrawn volume is replaced by buffered HBSS) is generally performed. However, the ***transfer-experiments*** (moving the filter insert from time point to time point from one HBSS-containing well to the next) can be necessary, for very rapidly transported substances ($P_{app} > 10^{-4}$ cm s^{-1})
- Volumes used for 12 mm-filters during the experiment: basolateral 1.2 mL apical 0.4 mL (24 mm-filters basolateral 2 mL, apical 1.5 mL)
- Incubations are done at 37°C under shaking (orbital shaker 500 rpm) to minimise the unstirred water layer
- Start (t =0)the washing solutions are removed and the filter inserts transferred into new wells containing the basolateral solution. Afterwards the apical solution is added
- Sampling:

Replacement experiments: when samples were taken, the volume is replaced by pre-warmed buffered HBSS

- when testing **ab transport**: in general, half of the volume in the receiver chamber is taken as sample
- when testing **ba transport**: the same sampling procedure as for ab transport is used
- **time-points:**
  generally: 15 min; 30 min; 45 min; 60 min
  but may be adjusted if:

  a. the substance is transported rapidly: shorter sampling times (e.g. 5 min, 10 min, 20 min, 30 min)
  or
  b. the substance is transported very slowly: longer sampling times (e.g. 30 min, 60 min, 90 min, 120min.)

A final sample from the donor chamber is taken to be able to calculate the mass balance (substance amount transported + substance amount left in donor / substance amount in donor at t=0).

A poor mass balance value after an experiment can indicate, that:

a. metabolism has occurred, or
b. that the compound is adsorbed in significant amounts to the experimental device (plastic of the 12-well plate, filter device or filter itself), or

c. that the compound is accumulated inside the cells.

- concentration at time point 0 (C_0): *for rapidly transported substances, take the C_0-sample from the vial with the donor solution instead of from the apical side of the monolayers, to minimize errors*

2.3 Deviation from the standard procedure

If the mass balance is poor, the compound is generally lipophilic and has a high clogD value, resulting in high adsorption to plastic. In this case the transport buffer in the basolateral chamber can be modified by, e.g. HBSS-Hepes + 4% BSA.

The transport experiment is followed by a BSA precipitation step. This precipitation is done by adding 2 volumes of acetonitrile to one volume sample, vortexing for 5 s and centrifugation for 10 min at 12000 rpm = 15000 g (Eppendorf-centrifuge 5403 with fixed angle rotor 16F24-11). Standards for the analytics are prepared in the 4%BSA HBSS-Hepes and handled by the same precipitation step. Also C_0 and final samples undergo the precipitation step.

Endpoint Measurement

The details of the analytical measurment are not covered by this protocol as they will be different for every compound tested. Depending on the compound, one of the following analytical procedures can used:

- UV-spectrophotometry (direct in HBSS solution)
- radiolabel [^{14}C] or [^{3}H]
- HPLC with UV-detection
- HPLC-MS/MS

Samples analysed with HPLC-MS might need to be diluted before analysis to prevent detector overflow; especially the donor solutions (C_0 and final donor samples)

The appropriate analytical method for the test compound should have a detection sensitivity within a broad relative dynamic rage of 1-1000, in order to quantify the wide range of fluctuations in the concentration of the compound in different compartments and various sampling points of the assay.

Acceptance Criteria

Quality Controls

1. Cell culture

Perform a Mycoplasma test every six months.

After 5 passages, verify that number of dead cells is less than 5% of the number of total cells before seeding.

2. Integrity control of Caco-2 monolayers grown on filter supports:

2.1 Mannitol permeability

At regular intervals (e.g. first cell passage seeded on filters after cells were thawed), the monolayer integrity is checked by measuring the permeablity of that paracellular marker (“negative control”).

Transport (ab) $P_{app} \geq 2 \times 10^{-7}$ cm s^{-1} (the exacty value is dependent on the FCS-batch used in cell culture).

Integrity-control of the monolayers by measuring the P_{app}-values for mannitol is done in parallel with the experiment to assess the influence of a compound on cell-monolayer integrity (“toxicity-assessment”; see section "Test Material Exposure Procedures").

2.2 TEER-values

TEER values are routinely measured before starting the experiments (after washing of the cell monolayers) using the Endohm tissue resistance measurement chamber (at 37 °C). Additionally, TEER is measured at the end of the transport experiment.

Test Acceptance Criteria

1. All tests

The TEER-values of our monolayers should exceed 150 Ω cm^2 to be acceptable [TEER values above 150 Ω cm^2 correspond to P_{app} values for [^{14}C]-mannitol < 5 x 10^{-7} cm s^{-1}]. TEER is additionally measured after the experiment is finished (after exchange of the HBSS-solution).

The mass balance should exceed 70%; at a lower mass balance-value, the P_{app}-value obtained gives only a rough estimation.

2. Toxicity tests (testing the effect of the test compound on the integrity of the monolayer, see section "Test Material

Exposure Procedures")

P_{app} values for [^{14}C]-mannitol up to 5 x 10^{-7} cm s^{-1} are accepted as normal. P_{app} values higher than 5 x 10^{-7} cm s^{-1} indicate that the cell-monolayer integrity is compromised. Compounds, which give rise to mannitol P_{app} > 10 x 10^{-7} cm s^{-1} are considered as “toxic” and a lower non-toxic donor concentration of the compound has be used for following tests.

3. Transport experiments (see section "Test Material Exposure Procedures"):

Special for lipohilic substances (clogD > 4.5):

If Papp-values are lower than 0.1x10^{-6} cm s^{-1}, the permeability of the compound across the filter without cell monolayer (filter pre-incubated in medium as if cell would be grown on it) shall be tested to exclude problems in the transport experiment created by adsorption to the experimental device (filter support).

Data Analysis

The change in donor concentration is taken into account after every sampling (i.e. the donor concentration recalculated);

1. Under sink conditions (the receiver concentration does NOT exceed 10% of the donor concentration) are given, the P_{app} [cm s^{-1}] can be calculated as follows:

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

where:

dQ/dt - the steady-state flux (dpm/s or µmol/s),
A - the surface area of the filter (cm^2) and
C_0 - the initial concentration in the donor chamber at each time interval (dpm/L or µM)

This formula is most commonly used for the calculation of the permeability coefficient Papp.

2. A general equation takes the “non-sink”-conditions into account, and makes the calculation of a correct permeability value possible even if the experiment does not exhibit linear flux (e.g. highly permeable substances). The advantage is that this analysis imposes less restriction on the experimental conditions.

In the “non-sink”- analysis, P_{app} is determined by non-linear curve fitting:

$$C_R(t) = [M / (V_D + V_R)] + (C_{R,0} - [M / (V_D + V_R)]) e^{(-Papp\, A\, (1/V_D + 1/V_R)\, t)}$$

where:

V_D - the volume of the donor compartment,
V_R - the volume of the receiver compartment,
A - the area of the filter,
M - the total amount of substance in the system,
$C_{R,0}$ - the concentration of the substance in the receiver compartment at the start of the time interval, and
$C_R(t)$ - the substance concentration at time t measured from the start of the time interval.

NOTE: A Microsoft-excel macro for calculating $\mathbf{P_{app}}$ using the non-sink equation can be provided on request by Johan Gråsjö (johan.grasjo@farmaci.uu.se)

Prediction Model

For Pharmaceutical compounds: see Artursson and Karlsson (1991) and Hubatsch et al (2007) and Prieto et al. (2010).

In the context of the ACuteTox project, Permeability Assay has been used together with other parameters to estimate the oral LD $_{50}$ dose. The full description of the analysis is currently in preparation.

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