In vitro permeability study

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Responsible teacher in the practical: Yan-Ru Lou

Checking the reports: Yan-Ru Lou
Cell culture

- Preventing contamination
  - Aseptic techniques: do not touch a sterile thing to a nonsterile surface; do not open a cell culture dish in unclean air
  - Culture one cell type at a time
- Be gentle to cells
- Check cells regularly under microscope
Cell counting

Count the cells within the large square and those crossing the edge on two out of the four sides.
The volume of 1 large corner square = $10^{-4}$ ml
Count 4 large corner squares and calculate the average.
If the average is 50, the dilution ratio is 1:2, the cell density in your cell solution is $50 \times 2 \times 10^4 = 10^6$ cells/ml.
MDCKII cells

MDCKII cells, Madin-Darby canine kidney cells (Canis familiaris; dog)
Culture of MDCKII cells

The cells grow in a few days to cover 80% of the surface area of a cell culture flask and they can be subcultured into new flasks or to permeable cell culture inserts.

Wash the cells with PBS and then incubate with TrypLE.

Growth conditions:
- Dulbecco’s Modified Eagle medium (DMEM), supplemented with 10% serum, penicillin-streptomycin and L-glutamine.
- +37°C, 95% relative humidity, 5% CO₂
- medium changed three times per week

Add the medium and centrifuge. Suspend the cell pellet in the medium and calculate cell number. Split the cells into new flasks.
Transwell systems used in the permeability study
Culture of MDCKII cells on the permeable supports

4.75 x 10^5 MDCKII cells are seeded onto 12-well plate polyester membrane inserts. Cell attachment takes about 1 day. Cell monolayer covers the surface area of the transwell. Permeability study 6 days after seeding.
Transwell

- Add medium into chamber below insert (basal side)
- Add medium into insert (apical side)
- Polyester membrane
- Chamber
- Medium fill level
- MDCKII cells

Permeability experiment using **lucifer yellow** and **propranolol** as reference compounds, **paracetamol** as a test compound

- **Lucifer yellow** has low permeability --- used to assess the integrity of the cell monolayer
- **Propranolol** has high permeability --- used as a highly permeable reference drug
- **HBSS-Hepes buffer, pH 7.4**
- **Drug concentrations:**
  - lucifer yellow and paracetamol at 250 µM
  - Propranolol at 50 µM
- **Drug transport direction** A → B
Cell seeding in inserts in a 12-well plate

5.10.2017

To be used for propranolol test
To be used for paracetamol test
To be used for lucifer yellow test

Faculty of Pharmacy/ Yan-Ru Lou
Medium change on Friday, Monday and Tuesday

6, 9, and 10.10.2017
Supervisors will change medium on weekends
Preparation of the permeability experiment

After medium change on Tuesday morning 10.10, each student prepares and labels these in the big lab 2060a. They can be found on the table in front of the fume hood:

- Seven 12-well plates
- 15-ml tubes for standard dilutions
- Eppendorf tubes for samples
- One black and one white 96-well plates

Keep them in your own work space on those tables reserved for teaching
Permeation study day

11.10.2017

Morning:

Prepare serial dilutions for three standard curves
Prepare and perform permeability test of propranolol

Lunch break

Afternoon:

Prepare and perform permeability tests of lucifer yellow and paracetamol
Add samples into two 96-well plates
Measure lucifer yellow samples
Send your UPLC sample layout (the Excel form) to Timo by email (timo.j.oksanen@helsinki.fi)
In the morning, preparing test solutions

- Ready 500 ml transport buffer: HBSS-10 mM Hepes (pH 7.4)
- Prepare test solutions:
  1. 250 µM lucifer yellow in transport buffer (in dark)
  2. 50 µM propranolol in transport buffer
  3. 250 µM paracetamol in transport buffer
- Warming transport buffer at 37°C water bath
Preparation of serial dilutions for standard curves

For example:

<table>
<thead>
<tr>
<th>Name of solution</th>
<th>Amount of 100 µM Pa (ml)</th>
<th>Amount of Transport Buffer (ml)</th>
<th>Pa concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pa1</td>
<td>7.5</td>
<td>2.5</td>
<td>75</td>
</tr>
<tr>
<td>Pa2</td>
<td>3.75</td>
<td>6.25</td>
<td>37.5</td>
</tr>
<tr>
<td>Pa3</td>
<td>0.75</td>
<td>9.25</td>
<td>7.5</td>
</tr>
<tr>
<td>Pa4</td>
<td>0.15</td>
<td>9.85</td>
<td>1.5</td>
</tr>
<tr>
<td>Pa5</td>
<td>0.075</td>
<td>9.925</td>
<td>0.75</td>
</tr>
<tr>
<td>Pa6</td>
<td>0.015</td>
<td>9.985</td>
<td>0.15</td>
</tr>
<tr>
<td>Pa7</td>
<td>0.005</td>
<td>9.995</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Everyone makes 3 standard curves: lucifer yellow, propranolol, and paracetamol
Propranolol permeability test

Start the timer

Add propranolol test solution

Keep 30 s interval between additions of test solution

Empty a insert, transfer it to the new well

To be used for propranolol test

To be used for paracetamol test

To be used for lucifer yellow test

0.5 ml

0.5 ml

0.5 ml

0.5 ml

Propranolol-5min

Propranolol-10min

Propranolol-15min

1.5 ml buffer

1.5 ml buffer

1.5 ml buffer

1.5 ml buffer

A

1

2

3

4

No cells

cells

cells

cells

B

cells

cells

cells

C

cells

cells

cells

Propranolol
Lucifer yellow and paracetamol permeability test

To be used for lucifer yellow test
To be used for paracetamol test
To be used for propranolol test

Start the timer
Add lucifer yellow test solution
Add paracetamol test solution
Keep 30 s interval between additions
Empty a insert, transfer it to the new well

Lucifer yellow-30min
Paracetamol-30min
TEER measurement

![TEER Measurement Diagram]

- **STX2 electrode**
- **Touching the bottom of the well**
- **WRONG WRONG**

Fig. 4—Angle Variance

Fig. 5—Depth Variance

**World Precision Instruments**

**EVOM**

**Current-passing electrodes**

**Voltage-measuring electrodes**

**EVOMX**
Lucifer yellow is measured by fluorometry Varioskan Flash (Thermo Fisher Scientific)

Excitation 425 nm and Emission 538 nm

200 µl of samples in a black 96-well plate
• Propranolol and paracetamol
• 200 µl of samples in an UPLC white 96-well plate
• Remember to label your plate with your name and date.
• Fill in the UPLC plate map (an Excel form is available).
Supervisors

Cell work supervisors

Alma  Mariia  Heikki  Jasmi

Yan-Ru  Patrick  Petter

Analysis supervisor

Timo
<table>
<thead>
<tr>
<th>Time</th>
<th>Event</th>
<th>Mon</th>
<th>Tue</th>
<th>Wed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MB, JK Medium change</td>
<td>MB, JK</td>
<td>MB, JK</td>
<td>MB, JK</td>
</tr>
<tr>
<td></td>
<td>MB, JK Saturday evening</td>
<td>MB, JK</td>
<td>MB, JK</td>
<td>MB, JK</td>
</tr>
<tr>
<td></td>
<td>MB, JK YRL</td>
<td>MB, JK</td>
<td>MB, JK</td>
<td>MB, JK</td>
</tr>
<tr>
<td>12:15-14:15</td>
<td>PS, HS Ida</td>
<td>PS, HS</td>
<td>PS, YRL</td>
<td>PS, YRL</td>
</tr>
<tr>
<td></td>
<td>PS, HS Emilia</td>
<td>PS, HS</td>
<td>PS, YRL</td>
<td>PS, YRL</td>
</tr>
<tr>
<td>14:15-16:15</td>
<td>PS Sampo</td>
<td>PS Sampo</td>
<td>PS Sampo</td>
<td>PS Sampo</td>
</tr>
</tbody>
</table>

**5-11.10.2017**

**Teachers:**
- YRL: Yan-Ru
- MB: Mariia
- HS: Heikki
- PS: Petter
- PL: Patrick
- AK: Alma
- JK: Jasmi

**7 students:**
- 4.10: add medium to transwells by JK
- 4.10: prepare cell lab by MB
- 4.10: prepare permeation lab by PS
- 4.10: prepare transport buffer by YRL
Data processing and results

- Raw data, three standard curves, and original calculations are presented in Microsoft Excel documents.
- Plot the accumulated test compound in the basolateral chamber versus time in Microsoft Excel documents.
- The average TEER values per insert before and after drug test are presented in the Appendix of the Microsoft Word report.
- Papp per insert is calculated and their average and SD are presented in Microsoft Word report.
- Mass balance is presented in Microsoft Word report.
Report instruction

Report is written in English using Microsoft Word.

1. Title
2. Author’s name
3. Introduction: 1-2 pages including the background and the aim of the study
4. Materials and Methods: no more than 3 pages
5. Results: present only the calculated results, including
   q Papp of each transwell in figures
   q the average Papp and standard deviations in a table
   q mass balance of each transwell in a table
6. Discussion: interpret the results, explain the possible reasons for the wired results, compare obtained data with the literature data, improvement of the study, future perspective
7. Conclusion:
8. References:
9. Appendix:
   q average TEER value per insert before and after tests in a table
Rules

• Be on time. If you miss or come late for more than 30 min to any session without good reason, you will not get credit.

• For permeation study:
  - Bring your own pen, calculator, the handbook, and memory USB stick
  - Bring your own lab coats

• For cell lab work:
  - Wear non-high heel shoes
  - Tie up your long hair

• Deadlines for submission of your report in Word document and Excel raw data with calculations: submit your report to Yan-Ru Lou (yan-ru.lou@helsinki.fi)
  - 27.10 Friday at 23:59
Errors to be corrected in the handbook

- Page 18, first table, Pa4, the amount of transport buffer should be 9.85 ml, not 9.855.
- Page 18, 2) Standard dilutions of Lucifer yellow (LY) for standard curve:
  \[ 20 \mu M \text{ LY} = 10 \mu l \text{ of } 20 \text{ mM LY stock} + 9.99 \text{ ml of Transport Buffer} \]
- Page 19, 2.5. Centrifuge the cell suspension at 1000 rpm - 200 rcf for 5 minutes
- Page 19, 2.7. Count the cells by using 0.1% Erythrosine B-dye
- Page 20, step 1.3. Wash the cell monolayers with prewarmed (37°C) transport buffer by adding 0.5 ml buffer to the apical side and 1.5 ml to the basolateral side of the monolayers.
- Page 20, step 2.7. Once you finish the measurements, wash the electrodes thoroughly first with MilliQ-H$_2$O and then with 70% EtOH.
- Page 26, \( P_{\text{app}} = \frac{\text{d}M/\text{dt}}{A \times (C_1-C_2)} \)