

Automation for Drug Transport Assay using 96-well Caco-2 filter plate

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Abstract:

The transport of drugs across the intestinal epithelial cell barrier is a major determinant factor of *in vivo* bioavailability. The human colon adenocarcinoma cell line (Caco-2) represents a well-established *in vitro* absorption model. A drastically increased number of new chemical entities are generated by combinatorial chemistry, requiring high throughput methods for Caco-2 absorption screening. Our laboratory has developed the automation of a 96-well format Caco-2 assay and validated a new integrity marker to allow higher throughput for permeability tests.

Introduction:

The clinical development of new drugs has often been stopped because of unfavourable pharmacokinetic properties other than lack of efficacy, animal toxicity, commercial or other reasons. ADME properties are now determined earlier in the process of drug development. The transport of drugs across the intestinal epithelial cell barrier is a major determinant factor of *in vivo* bioavailability.

The human colon adenocarcinoma cell line (Caco-2) represents a well-established *in vitro* absorption model. This model has been widely utilized to obtain early prediction of oral bioavailability for compounds known to possess desired biological activity, accelerating the identification of lead compounds. Currently, a drastically increased number of new chemical entities are generated by combinatorial chemistry, requiring high throughput methods for Caco-2 absorption screening.

To assess monolayer integrity (in each well of the plate), three protocols can be used : Transepithelial Electrical Resistance measurement, Lucifer Yellow or ¹⁴C-mannitol permeability. To simplify and enable automation of Caco-2 assay in 96-well plates, we have developed a monolayer integrity marker which can be simultaneously analyzed with the tested compounds in the same sample by liquid chromatography with tandem mass spectrometry detection (LC-MS/MS). For this purpose atenolol, with absorption properties similar to those of mannitol, is used.

We here describe an assay on Caco-2 cells in Multiscreen Caco 96-well plate from Millipore on a Caliper LifeSciences Staccato automation platform.

Picture 1 - Detail of the Caco-2 automation platform:

- A: 96-pipette Head - Sciclone ALH;
- B: Microplate Handler: Twister II;
- C: Incubator at 37°C;
- D: Lidded 96-well Caco-2 Plate



Methods:

Cell culture : Caco-2 cells were routinely maintained in MEM supplemented with 10% serum. Cells were seeded in 75 µL at 9000 cells/well in apical compartment in the Multiscreen Caco 96-well plates. Cells were incubated at 37 °C in 5 % CO₂ for 21 days for differentiation. Medium was changed every two days.

Research of membrane integrity marker : ¹⁴C mannitol and atenolol were co-incubated on Caco-2 cells and permeability tests were performed on these compounds.

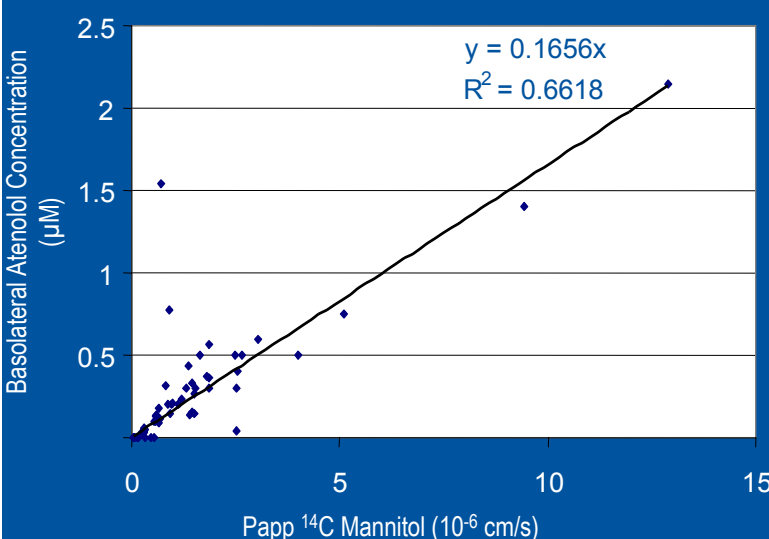
Permeability test : On the automation platform, plate was disassembled, medium was removed in apical and basolateral compartments and HBSS buffer was added in both compartments. After pre-incubation at 37 °C for 20 min and medium aspiration, compounds (10 µM) and membrane integrity marker in HBSS buffer (containing MES 2.5 mM, pH=6.5) in a total volume of 75 µL were added in apical compartment. HBSS buffer (containing HEPES 5 mM, pH=7.5) was added in basolateral compartment (250 µL). The Caco-2 plate was then incubated for 2 hours at 37 °C in 5 % CO₂. Drug samples and atenolol were analyzed using LC-MS/MS with Turbo Ionspray source method.

Materials:

Caliper LifeSciences Staccato system included :

- Liquid Handler: CaliperLS Sciclone ALH 500 equipped with a High Volume 96 tip Head, Z8 and Gripper
- Incubator: Kendro CO₂ Cytomat 6000
- Plate handler and Stacker: CaliperLS Twister II
- Scheduler: Clara software
- Detection: Quattro Micro spectrometer (Waters)
- Microplates: Multiscreen Caco 96-well plate from Millipore
- Cell culture : Caco-2 cells from ECACC

Results:



Graph 1- Atenolol – Mannitol permeability Correlation

	AUTOMATED METHODOLOGY									MANUAL	
	Plate 1			Plate 2			Plate 3			METHODOLOGY	

Compound	Theor. Conc (µM)	N	Mean Papp (10 ⁻⁶ cm/s)	SD	N	Mean Papp (10 ⁻⁶ cm/s)	SD	N	Mean Papp (10 ⁻⁶ cm/s)	SD	Mean Papp (10 ⁻⁶ cm/s)	Permeability Classification	Fabs (%)
ranitidine	10	12	0.4	0.1	12	0.3	0.1	6	0.3	0.1	0.8	Low	50
furosemide	10	12	0.0	0.0	11	0.8	1.3	8	1.6	2.6	1.0	Low	61
terbutaline	10	12	0.4	0.1	7	1.4	2.4	11	1.1	3.3	2.3	Low	70
guanabenz	10	12	27	1.5	5	21	0.9	14	23	2.3	58	High	75
caffeine	10	12	48	1.3	5	50	2.5	6	51	3.8	53	High	100
antipyrine	10	24	45	1.2	21	48	1.8	7	43	16	47	High	98

Table 1 – Reproducibility study of 6 known compounds.

Two permeability classes defined: apparent permeability (Papp) greater or equal to 3.10⁻⁶ cm/s corresponds to "High" class and thus predicts a high absorption fraction in humans.

Discussion:

Comparison between atenolol and mannitol permeability shows a good correlation (Graph 1). Atenolol can be used as an alternative to mannitol for membrane integrity marker; thus avoiding use of radioactivity and allowing compound analysis and integrity assay in the same LC-MS/MS run.

Classification for every studied compound is unchanged between automated and manual methodologies. Repeatability and reproducibility of permeability values were satisfactory with automated method.

Permeability testing throughput can be increased by the automation of the Caco-2 test in 96-well format in a reliable manner, using LC-MS/MS for compounds and integrity marker analysis.