Automated Absorption Assessment Using Caco-2 Cells Cultured on Both Sides of Polycarbonate Membranes

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Received May 25, 1998; accepted December 1, 1998

Purpose. To increase the capacity of *in vitro* absorption assessment and to decrease the amount of substance needed to perform early mechanistic investigations.

Methods. A liquid handling system, combined with a shaker and heating plates, was used to automate the Caco-2 cell based *in vitro* absorption assessment assay. In order to decrease the amount of substance needed for early mechanistic studies, a method for culturing Caco-2 cells on the lower side of polycarbonate membranes was also developed.

Results. Similar results were obtained with the automated assay as compared to manually performed assays. Data presented suggest that active transport and efflux were decreased in cells cultured on the lower side of the membranes as compared to ordinary seeded cells.

Conclusions. Implementation of a liquid handling system for *in vitro* absorption assessment as reported here decrease the manual workload and increases the capacity of this *in vitro* assay substantially. Caco-2 cells cultured on the lower side of polycarbonate membranes, as described in this article, can not be used for analysis of transport mechanisms.

KEY WORDS: Caco-2 cells; automated absorption assessment; cell culture; transport; permeability; polycarbonate membranes; upper and lower side.

INTRODUCTION

During the last few years there has been an increased need for absorption assessment in drug discovery (1,2). High throughput screening assays and combinatorial chemistry, together with an increasing awareness of the importance of early predictions of bioavailability during drug discovery, has increased the number of substances to be evaluated extensively. Assessment of absorption is one essential part in the prediction of bioavailability. For orally administered compounds permeability through Caco-2 cell monolayers correlate well with *in vivo* absorption in man (3,4) and has become a well established *in vitro* method (5). The use of *in vitro* models increase the capacity for absorption assessment substantially as compared to *in vivo* studies and has also decreased the amount of substance needed. However, manually performed assays are still too tedious and labor consuming to meet the increased demand in early phases of drug discovery, and the amount of substance needed for absorption assessment in these early phases has to be decreased even further.

In order to increase the capacity for early screening of absorption potential we have now applied an automatic sample processor for the assessment of permeability *in vitro* and here report an evaluation of this automated assay. Furthermore, to decrease the amount of test compounds needed for analyzes of transport mechanisms and to simplify the processing of different test protocols, we also present a method for culturing of Caco-2 cells on the lower side of polycarbonate membranes and an evaluation of this method is also presented in this paper. At present, experimental conditions are not the same when studies on apical to basolateral and basolateral to apical transport are performed, since the volumes used in the membrane inserts and wells are not the same. Another advantage of using cells cultured on the lower side of the membranes would therefore be, that the same experimental conditions could be used for studies on transport in both the apical to basolateral and the basolateral to apical direction.

MATERIALS AND METHODS

Materials

Labeled test-compounds were purchased from Dupont NEN Life Science Products, Belgium, ^{14}C-mannitol (51.5 mCi/mmol) and ^{3}H-testosterone (53.5 Ci/mmole) or Amersham Life Science, UK, ^{3}H-vinblastine (16.0 Ci/mmole) and ^{3}H-L-phenylalanine (54.0 Ci/mmole). All materials used for cell culturing were obtained from Life Technologies, Sweden. Medium and buffers where originally from GIBCO and cell culture plastics from Costar. All other chemicals used where of at least analytical grade and obtained from local suppliers.

Experimental Set-Up

The automated set up (Fig. 1) consisted of a liquid handling system, MultiPROBE 204 (Packard Instrument Company, Meriden, CT) performing all necessary pipetting steps during the permeability assay. Permeability experiments were performed on the work surface of the MultiPROBE, at 37°C and 300 rpm on a heating plate (TH 60 SMZ warm stage, Linkam Scientific Instruments LTD, Tadworth, Surrey, UK) mounted on a shaker (Micromix 5, Diagnostic Products Corporation, Los Angeles, CA). The shaker was controlled from the assay program of the MultiPROBE and was stopped at all aspirating and dispensing steps. Scintillation vials (6 ml) were placed in a 16 mm tube rack with 192 positions (Packard Instrument Company). Two heating plates could be attached to the shaker and each heating plate could accommodate two 12 well plates, giving a maximal capacity of four plates in each assay. Programming of the liquid handling system was performed using the visual basic software, MPTable 3.7 (Packard Instrument Company). All buffer used in the assays was prewarmed in a reservoir that was heated to 37°C by a recirculating waterbath.

In order to fixate the membrane inserts during shaking, and to decrease evaporation, in-house designed lids were used (Fig. 2). Holes (10 mm in diameter) were drilled in standard lids from 12 well plates, and were positioned above one side of each well, so that the MultiPROBE could get access to both inserts and wells during sampling. Two metal pins (4 mm each) were placed close to each hole on the inside of the lid, in such a way that the inserts became fixated during shaking. During manually performed assays, cells were incubated on a shaker in a humidified incubator at 37°C as described previously (5).

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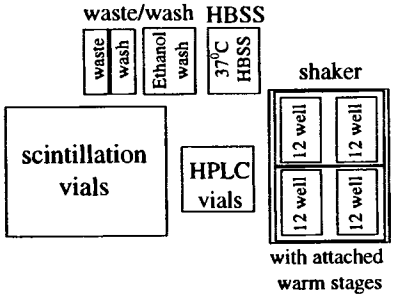

Fig. 1. Automatic absorption assessment. Schematic drawing of the work surface of the MultiPROBE 204, containing a shaker with two attached warm stages, on which four cell culture plates can be incubated. Placement of two different racks for sample vials (scintillation and HPLC vials) is also illustrated. The wash and waste position contains two wash bowls, one for water and one for ethanol. Prewarmed Hanks balanced salt solution is kept in the beaker depicted HBSS which is connected to a recirculating water bath (not shown).

Cell Culture

The human colon adenoma derived cell line, Caco-2, was obtained from ATCC (HTB-37) and cultured as described by Artursson et al (5). Cells used in the screening assay (passage 36–46) were plated on 12 mm diameter polycarbonate membranes, 0.45 μm pore diameter (Transwell, Corning Costar, Acton, MA), at a density of 0.5×10^6 cells/membrane. Experiments were performed within 2–4 weeks after seeding the cells on the membranes, or as stated in the text.

For seeding of cells on the lower side of the membranes, membrane inserts were turned upside down and 350 μl cell-suspension was added to each membrane (0.5×10^6 cells/membrane). After addition of the cells, membranes were incubated in a large petri dish (150 mm in diameter) containing 50 ml DMEM, for three to four hours. After this attachment period, the membranes were turned the right way around and placed in 12 well plates and incubated in the same way as ordinary seeded membranes.

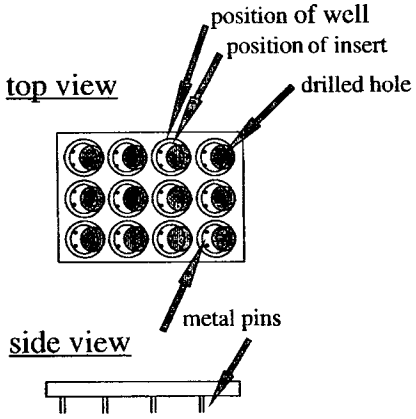

Fig. 2. Lid design. In-house designed lids were made by drilling holes (10 mm in diameter) in ordinary lids obtained from 12 well plates. The holes were positioned above one side of each well, so that the MultiPROBE could get access to both the insert and the well during sampling. Two metal pins (4 mm each) were placed close to each hole on the inside of the lid, in such a way that one of the "arms" of the inserts could be placed between the pins, in order to fixate the inserts (placement of the pins are marked with black dots).

Permeability Assays

Permeability assays were performed essentially as described in (5) using either the automatic or manual set up as stated in the text. Cells were rinsed once and incubated in Hanks balanced salt solution containing 25 mM HEPES, pH 7.2 (HBSS), 450 μl in inserts and 1500 μl in the wells. Integrity of individual monolayers was monitored during experiments by addition of labeled mannitol to all test solutions, either alone or in the presence of additional test compound as indicated. The volume added to the membrane inserts was increased to 500 μl, when transport of test substances from wells to inserts was assayed. Samples were taken during a two hour incubation, at 15, 30, 45, 60, 90 and 120 min and replaced with fresh pre-warmed HBSS. Ultima Gold scintillation cocktail (Packard Instrument Company) was added to the samples and the activity determined using a liquid scintillation counter (Tri-Carb 2700TR, Packard Instrument Company). The apparent permeability (P_{app}) was calculated as described previously (5). All experiments were performed using quadruplicate membranes and statistical analysis was made using Student's t-test. Monolayers with a P_{app} for mannitol higher than 0.5×10^{-6} cm/s were not used for calculations and are thus not included in the data presented in Tables I and II.

Morphological Studies

At different time points after seeding, membranes with cells growing on either the upper or lower side were taken and assayed for: 1) viability, 2) total cell number, and 3) tight junctions (by staining for actin). All staining were performed as described in (5). Cell counts were made on photographs taken. One randomly chosen area, corresponding to 0.01 mm^2, were counted on three different filters from each group and the mean and standard deviation were calculated. Electron microscopy was also used to compare the morphology of cells grown on different sides of the membranes (6).

Integrity Studies Using ^{14}C-mannitol

The permeability of ^{14}C-mannitol was used to compare integrity of cell monolayers, grown on different sides of the membranes, at different time points after seeding. The permeability assay was run as described above, and were performed both at permissive (2–4 weeks after seeding) and non-permissive (earlier or later) time points after seeding.

RESULTS

Comparison Between Manual and Automated Permeability Assay with Cells Grown on the Upper Side of the Membranes

As can be seen from Table I results obtained from the automated assay were similar to those obtained from manually performed assays. Permeability of mannitol, which is passively transported via the paracellular route and which commonly is used as a marker of monolayer integrity, was almost identical in the two assays, both for transport in the apical to basolateral

Table I. Comparison Between Manual and Automated Permeability Assays, Using Cells Grown on the Upper Side of the Filters

Test substance	Direction	$P_{app} \times 10^6$ (cm/s) Manual	$P_{app} \times 10^6$ (cm/s) Automated	P
Mannitol	Ap>Bl	0.17 ± 0.08 (n = 26)	0.14 ± 0.03 (n = 36)	< 0.05
	Bl>Ap	0.16 ± 0.06 (n = 20)	0.15 ± 0.04 (n = 34)	ns
	P	ns	ns	
Testosterone	Ap>Bl	89 ± 12 (n = 11)	80 ± 16 (n = 12)	ns
	Bl>Ap	87 ± 6.4 (n = 11)	77 ± 12 (n = 6)	< 0.05
	P	ns	ns	
Vinblastine	Ap>Bl	1.0 ± 0.50 (n = 23)	1.6 ± 0.84 (n = 20)	< 0.01
	Bl>Ap	26 ± 3.5 (n = 24)	31 ± 4.4 (n = 20)	< 0.001
	P	< 0.001	< 0.001	

Note: Automated or manual permeability assays were performed with Caco-2 cells cultured on the upper side of polycarbonate membranes and the apparent permeability calculated (P_{app}). Each of the labeled test substances were assayed at 2–10 different occasions and mean ± SD were calculated (n = total number of membranes used). The significance of the difference between the means (either Ap > Bl as compared to Bl > Ap direction, or Manual as compared to Automatic assay) were tested using the Student's t-test. P is the level of significance (ns = non-significant).

and basolateral to apical direction. Results with testosterone were also similar in the two assays, and non of the assays detected any differences between the two directions. The actively excreted substance vinblastine had a somewhat higher permeability in both directions in the automated assay, but a pronounced polarized efflux was still observed. The ratio between basolateral to apical and apical to basolateral transport was 26 and 19 times for the manual and automatic assay respectively.

Characterization of Cells Cultured on the Lower Side of the Membranes

Cell densities were found to be the same in monolayers cultured on the upper and lower side of the membranes, both after 14 days (1.22 and 1.24 × 10^6 cells/cm^2 respectively) and 26 days after seeding (1.17 and 1.16 × 10^6 cells/cm^2 respectively). The viability was estimated to be higher than 99%, irrespectively if the monolayers had been grown on the upper or lower side of the membranes. Occasionally clusters of dead cells were seen, but there was no difference in the number of clusters in cells grown on the lower side as compared to cells grown on the upper side. Staining for actin, revealed a similar and typical perijunctional staining in cells grown on the upper as well as the lower side of the membranes, indicating a well developed tightness of the junctions between the epithelial cells (not shown). Using transmission electron microscopy it was found that cells grown on the lower side of the membranes had the same cellular size, expression of microvilli, tight junctions and a similar degree of well organized terminal web (not shown) as reported previously for well differentiated Caco-2 cells (6).

As evidenced from mannitol permeability the integrity of monolayers grown on the upper and lower side of the membranes were similar 14 days after seeding, 0.28 ± 0.15 ×

Table II. Comparison of Automated Permeability Assays Performed with Cells Grown Either on the Upper or Lower Side of the Filters

Test substance	Direction	$P_{app} \times 10^6$ (cm/s) Upper side	$P_{app} \times 10^6$ (cm/s) Lower side	P
Mannitol	Ap>Bl	0.14 ± 0.02 (n = 28)	0.19 ± 0.11 (n = 22)	< 0.05
	Bl>Ap	0.15 ± 0.03 (n = 25)	0.11 ± 0.04 (n = 21)	< 0.001
	P	ns	< 0.01	
Testosterone	Ap>Bl	80 ± 16 (n = 12)	86 ± 6.3 (n = 8)	ns
	Bl>Ap	77 ± 12 (n = 6)	65 ± 2.3 (n = 4)	ns
	P	ns	< 0.001	
Vinblastine	Ap>Bl	1.6 ± 0.97 (n = 15)	2.5 ± 1.3 (n = 15)	< 0.05
	Bl>Ap	30 ± 4.8 (n = 14)	21 ± 6.5 (n = 15)	< 0.001
	P	< 0.001	< 0.001	
L-Phenylalanine	Ap>Bl	14 ± 2.9 (n = 12)	7.1 ± 1.9 (n = 9)	< 0.001
	Bl>Ap	5.5 ± 1.9 (n = 12)	5.3 ± 1.3 (n = 9)	ns
	P	< 0.001	< 0.05	

Note: Automated permeability assays were performed with Caco-2 cells cultured on either the upper or lower side of polycarbonate membranes and the apparent permeability calculated (P_{app}). Each of the labeled test substances were assayed at 1–7 different occasions and mean ± SD were calculated (n = total number of membranes). The significance of the difference between the means (either Ap > Bl as compared to Bl > Ap direction, or upper side as compared to lower side) were tested using the Student's t-test. P is the level of significance (ns = non-significant).

10^{-6} cm/s and 0.32 ± 0.11 × 10^{-6} cm/s respectively. The permeability of mannitol was also found to be the same 21 days after seeding, while it was higher in cells grown on the lower side 28 days after seeding, 1.3 ± 0.26 × 10^{-6} cm/s as compared to 0.35 ± 0.07 × 10^{-6} cm/s (n = 4 filters for each mean, $p < 0.001$ at 28 days, while no statistical difference was detected after 14 days). In addition, the variability in mannitol permeability between different filters was usually larger when cells had been grown on the lower side of the membranes. The largest variability was seen before and around 14 days after seeding, and after and around 28 days after seeding, while monolayers grown on the lower side for three weeks were less variable. When membranes were examined earlier than 14 days after seeding, it was found that the integrity of monolayers grown on the lower side increased more slowly with time in culture, as compared to monolayers grown on the upper side.

Transport Across Monolayers Cultured on the Upper or Lower Side of Membranes

Mannitol and testosterone gave results which is normal for passively transported compounds, both when monolayers grown on the upper and lower side were used. As can be seen in Table II no significant difference was detected when permeability's in both directions were compared in cells grown on the upper side. While a significant difference between the two directions was detected in cells grown on the lower side, for both mannitol ($p < 0.01$) and testosterone ($p < 0.001$). These differences were quite small (between 30 and 40%) and mannitol as well as testosterone would therefore have been considered to be passively transported compounds, disregarding if cells grown on the upper or lower side had been used.

Active excretion and transport was on the other hand higher in monolayers grown on the upper side, as compared to cells grown on the lower side, and this difference was seen disregarding how long the cells had been grown after seeding. The P_{app} for vinblastine was about twenty times higher in the basolateral to apical direction, as compared to the apical to basolateral direction, for cells grown on the upper side ($p < 0.001$), and this difference was only about eight times in cells grown on the lower side ($p < 0.001$). As can be seen from Table II, P_{app} in the basolateral to apical direction was significantly lower in cells grown on the lower side ($p < 0.001$), while P_{app} in the apical to basolateral direction seemed to be higher ($p < 0.05$). For cells grown on the upper side, phenylalanine had a three times higher permeability in the apical to basolateral direction, as compared to the basolateral to apical direction ($p < 0.001$), indicating active transport of this amino acid, while this difference was very small (only about 1.3 times) in cells grown on the lower side ($p < 0.05$), suggesting passive transport in these cells.

DISCUSSION

From the data presented it can be concluded, that the automated set up performs absorption assessment with Caco-2 cell monolayers equally well as compared to manually performed assays. The automatic set up accommodates four 12 well plates and since two 2-hour experiments easily can be performed in one day, the capacity of the automatic assay would be 96 membranes per day. To calculate the number of substances that can be run each day, several aspects has to be taken into consideration; the number of replicates used for each substance; if data on both apical to basolateral, and basolateral to apical direction is wanted; the length of incubation used, which might affect the number of assays that can be run in one day; and if it is desirable to include one or more reference compounds. Using only two membranes for each compound, one for each direction, the maximum screening capacity using two hour incubations would be as high as 240 substances per week. While the use of quadruplicate membranes would decrease the number of compounds to 60 per week. It should also be pointed out that the use of short sampling time intervals (less than 45 min) would decrease the capacity of the set up.

As illustrated in Table III the automated set up decreases the manpower needed to perform absorption assessment considerably. The steps that at present still have to be manually performed are transport of cells from the cell culture laboratory to the MultiPROBE work surface and the handling of all sample vials. The MultiPROBE was programmed only to perform the first change of medium to HBSS in the inserts, while the initial change of medium to HBSS in the wells was manually performed, a step which also could be automated if desired. Different types of sample vials can be used and as seen in Fig. 1 the work surface of the MultiPROBE can accommodate and use different types of racks, for different sample vials, simultaneously. The flexibility of the system makes it easy to switch

Table III. Comparison of Manual and Automated Work Load During Automated Absorption Assessment

Operator tasks	MultiPROBE tasks
• place inserts in HBSS-containing wells	
• transport cells to work surface	
• start MultiPROBE	aspirate medium from inserts
•	add new HBSS
•	start shaker
•	stop shaker after 15 min.
•	aspirate HBSS from acceptor side
•	add new HBSS
•	aspirate HBSS from donor side
•	add test-solution
•	take samples from donor side
•	1) start shaker
•	2) stop shaker after xx min.
•	3) take samples from acceptor side
•	4) add same volume HBSS to acceptor side
•	repeat previous four steps N times
•	start shaker
•	stop shaker after xx min.
•	take samples from acceptor side
•	take samples from donor side
•	end
• remove samples	

Note: The manual workload (left column) consisted of transportation of cells to the work surface and removing the samples at the end of the assay. When started, the MultiPROBE performed all other necessary steps needed for the absorption assessment assay (right column).

between different sample vials and also facilitates the use of other cell culture plates if needed.

The use of Caco-2 cells grown on the lower side of membranes would decrease the amount of test substance needed for measuring basolateral to apical transport to 0.45 ml/filter, instead of 1.5 ml used at present. This would reduce the amount of compound needed more than 50%, when measurement of apical to basolateral and basolateral to apical transport is to be performed (0.45 + 0.45 ml as compared to 0.45 + 1.5 ml). The use of cells cultured on the lower side of the membranes for studies on basolateral to apical transport, would make experimental conditions similar to that used for studies on apical to basolateral direction (that is: 0.45 ml test solution and 1.5 ml acceptor solution in both cases), which is likely to simplify analysis and interpretation of test results. However, this method for culturing cells on the lower side of the membranes can not be recommended at present. The frequency of leaky ($P_{app} > 0.5 \times 10^{-6}$ cm/s) monolayers grown on the lower side was higher and the time frame after seeding within which monolayers with low variability in integrity could be obtained were decreased. As can be seen from Table II, the passively transported compounds mannitol and testosterone gave the same results in monolayers grown on the upper and lower side of the membranes, while active transport of phenylalanine and active excretion of vinblastine were lower in monolayers grown on the lower side. Phenylalanine would even have been detected as a passively transported compound using cells cultured on the lower side.

It can be concluded that it is possible to increase the capacity of *in vitro* absorption assessment extensively by using an automatic sample processor as presented here. In order to fully exploit the capacity of this automated set up the capacity of chemical analysis also has to be increased, a challenge that we and several other laboratories presently are working with. Results obtained using the automated assay with Caco-2 cells cultured on the upper side of polycarbonate membranes, were the same as those obtained by manually performed assays. The routine use of Caco-2 monolayers grown on the lower side of the membranes can however not be recommended at present. Continues work in this laboratory aims at making this possible in order to decrease the amount of substance needed for analyzes of transport mechanisms, and to simplify performance and evaluation of *in vitro* absorption assessment even further.

ACKNOWLEDGMENTS

We would like to thank Sheila Forsman, Vibeke Täpp, Veronika Wahlund and Carina Westberg for their skillful technical assistance during different parts of this study.

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