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Sodium glycocholate transport across Caco-2 cell monolayers, and the enhancement of mannitol transport relative to transepithelial electrical resistance

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Abstract

Ideally, the amount of enhancer remaining at the donor side during an in vitro transport study should be known, in order to know the true enhancer concentration during a permeability study. The purpose of the present study is to estimate the flux of the enhancer, sodium glycocholate (GC), through Caco-2 cell monolayers, and to study the effect of various enhancer concentrations on the permeability of GC itself, the permeability of mannitol and the transepithelial electrical resistance (TEER).

Apical to basolateral permeability was measured with various concentrations 0.50% (10.2 mM), 0.75% (15.5 mM) and 1.00% (20.5 mM) of GC. The GC permeabilities (Papp) were 4.7 ± 1.1, 12.8 ± 2.8 and 25.8 ± 4.3 ($\times 10^{-7}$ cm s^{-1}), respectively. Mannitol transport changed accordingly with the Papp; 8.5 ± 0.8, 9.9 ± 2.7, 20.4 ± 2.8 and 31.0 ± 4.9 ($\times 10^{-7}$ cm s^{-1}) for GR, 0.50, 0.75 and 1.00% GC, respectively, with a TEER after 120 min, relative to initial, of 86 ± 6, 77 ± 10, 61 ± 11 and 49 ± 7%.

In conclusion a low and concentration-dependent permeability was found for GC across the Caco-2 cells. Mannitol transport increased and TEER decreased accordingly with increasing GC concentrations. TEER decreased in less than 10 min to a certain level, without further reduction in a 120 min period, indicating that the enhancer effect is momentarily, rather than time-dependent. The apical GC concentration and enhancer effect may be considered well defined during the experiment, due to the observed low permeability of GC.

Keywords: Caco-2; TEER; Sodium glycocholate

1. Introduction

Ideally, the amount of enhancer remaining at the donor side during an in vitro transport study should be known. Alternatively, the enhancer may be added to the donor as well as the acceptor side as seen in Ussing chamber with mounted nasal tissue or Caco-2 cells (Jørgensen et al., 1993; Jørgensen and Bechgaard, 1994).

Knowing the exact enhancer concentration may be of importance for several reasons, especially in mechanistic studies, where the surfactant's critical micelle concentration (CMC) is of potential importance. The surfactants increase the permeability of epithelial barriers in a concentration-dependent manner, but the specific mechanism is still un-

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clear (Ward et al., 2000). Besides affecting the integrity of the tight junctions (Anderberg and Artursson, 1993), the surfactants may be incorporated into the lipid bilayer or solubilizing the lipid membrane.

In vitro study of enhancer transport has been performed by Bechgaard et al. (1993). The transport of didecanoyl-L-α-phosphatidylcholine across nasal tissue mounted in Ussing chambers was measured, primarily in order to predict toxicity due to systemic transport. The observed transport was very low (>about 1% h^{-1}).

The Caco-2 cell monolayer have been valuable in mechanistic absorption-enhancer studies (Noach, 1995; Ward et al., 2000), and today it is the most widely used in vitro method. For certain enhancers, a linear relationship is observed, between the enhancer effect and the cytotoxicity in Caco-2 cell monolayers (Quan et al., 1998). Whether it is possible to find an enhancer with better effect/toxicity ratio is not known, but a new class of enhancers, chitosans, has been reported promising (Aspden et al., 1997; Illum, 1998; Kotze et al., 1998).

The bile acid sodium glycocholate (GC) is an enhancer with relatively low toxicity (Gizurarson et al., 1990) and good enhancing properties (Pontiroli et al., 1989). As expected the GC toxicity and enhancer effect is dependent on the concentration. In Caco-2 cells Jørgensen et al. (1993), found a 50% reduction in intracellular dehydrogenase activity and about 100 times increase in mannitol transport at a concentration of 24.2 mM (1.2%). A concentration of 5.1 mM did not show toxicity nor enhancer effect. Therefore, the effective GC concentrations were found to be higher than the CMC (2–5 mM). Senel et al. (1998), studied the effect of GC on porcine buccal mucosa and found no enhancer effect of 10 mM. However, the transport of GC across the buccal epithelia was increased 5–6 times at a 100 mM enhancer concentration.

The purpose of the present study is to estimate the flux of the enhancer, GC, through Caco-2 cell monolayers, and to evaluate the effect of various enhancer concentrations on the permeability of GC itself, the permeability of mannitol and the transepithelial electrical resistance (TEER).

2. Materials and method

2.1. Materials

The Caco-2 cell line was obtained from the American Type Culture Collection (Rockville, MD, USA) at passage 19. The cells were seeded in culture flasks and passaged in Dulbecco's modified eagle's medium (DMEM) from Gibco laboratories (Lenenxa, KS, USA) supplemented with 10% fetal bovine serum from Biowhittaker (Walkerville, MD, USA). The media was added penicillin/streptomycin (100 U ml^{-1} and 100 μg ml^{-1}, respectively), 1% L-glutamine and 1% non-essential amino acids from Gibco laboratories.

Glucose-Ringer (GR) solution was prepared of 285 mg disodium hydrogen phosphate dihydrate, 55 mg sodium dihydrogen phosphate, 244 mg magnesium chloride hexahydrate, 176 mg calcium chloride dihydrate, 373 mg potassium chloride, 2100 mg sodium hydrogen carbonate, 6568 mg sodium chloride and 1802 mg D(+)-glucose dissolved in a total volume 1 l with distilled water. All Ringer components are of analytical grade purchased from Merck (Darmstadt, Germany), whereas glucose was from May and Baker (Dagenham, UK).

^{3}H-Mannitol with a specific activity of 20 Ci $mmol^{-1}$ were purchased from Sigma Chemicals (St. Louis, MO, USA). TEER was measured with a voltohmmeter (EVOM) and a fork electrode, both of which were from World Precision Instruments (Sarasota, FL, USA).

2.2. Preparation of Caco-2 monolayers

Cells were harvested by use trypsin–EDTA from Gibco laboratories and seeded onto polycarbonate filters (0.3 μm pores, 1 cm^2 growth area) at a density of 10^5 cells cm^{-2} inside Transwell cell culture chambers (Costar, Cambridge, MA, USA). Monolayer cultures were grown in an atmosphere of 5% CO_2–95% O_2 at 37 °C. Growth media were replaced every other day and cells from passage 23–25 were applied. Cells were used after 22 days of incubation on filters and all wells received new media the day before use. Before initiating the transport study the cells equilibrated 1 h with fresh made GR and the TEER was measured.

2.3. GC transport across filters and adsorption to chambers

The filter transport was examined by using a 0.02% GC solution in GR added to the apikal side and taking basolateral samples 5, 15, 30 and 60 min after ($n = 3$). The adsorption to empty chambers was performed by adding a 0.0005% solution to the chambers, whereafter samples were withdrawn 5, 15, 30 and 60 min after.

2.4. Enhancer transport study

Apical to basolateral permeability was measured with various concentrations 0% (control), 0.5% (10.2 mM), 0.75% (15.5 mM) and 1.00% (20 mM) of GC purchased from Sigma. The pH of the transport media was adjusted to 7.4; 0.5 ml of the transport media was added on the apical side ($n = 3$, for each of three passages) and 1.0 ml GR on the basolateral. Samples were taken from the apical side (20 μl without replacing the volume, diluted to 100 μl with GR) 5, 70 and 120 min and from the basolateral side (100 μl, with refill) 10, 20, 30, 40, 50, 60, 80, 100, 120 min after initiating the study. The samples were transferred to glass tubes (0.1 ml) from Aarhus Mikrolab (Aarhus, Denmark) for automated injection on HPLC and a volume of 50 μl was injected. To evaluate the basolateral stability samples were withdrawn from the chamber, after removing the insert, at 140, 160 and 180 min. Stability results are given as 180 min values relative to 140 min. TEER was measured after the basolateral sample withdrawal and refill was performed.

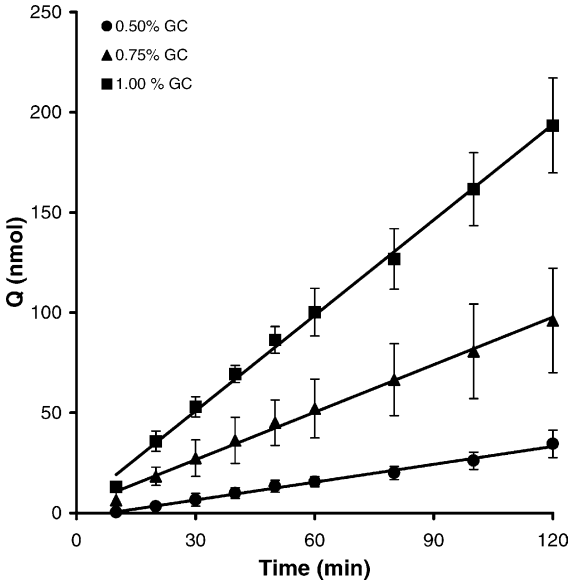

Fig. 1. Average transport profile for sodium glycocholate (GC) across Caco-2 cell monolayers ($n = 3$, for each of three passages). The initial apical GC concentration was 0.50% (10.2 mM), 0.75% (15.5 mM) and 1.00% (20.5 mM), respectively.

2.5. Mannitol transport

An amount of 0.5 ml of the transport media (same as above) was added on the apical side ($n = 3$, for each passage) and 1.0 ml GR on the basolateral. After diluting the ^{3}H-mannitol to a concentration of 44 μCi ml^{-1}, 20 μl was added in wells from the same passages as the GC transport study and samples were withdrawn from the apical side (20 μl without refill) and from the

Table 1
Sodium glycocholate (GC) transport, mannitol transport and TEER data (±S.D.) after application of various concentrations of GC on the apical side of Caco-2 cell monolayers ($n = 3$, for each of three passages)

Parameters	GC concentration			
	0% (GR) (0 mM)	0.50% (10.2 mM)	0.75% (15.5 mM)	1.00% (20.5 mM)
GC Papp cm s^{-1} × 10^7	–	4.8 ± 1.1	12.9 ± 2.8	25.8 ± 4.3
95% confidence interval	–	[4.0–5.6]	[10.8–14.0]	[22.5–29.1]
Mannitol Papp cm s^{-1} × 10^7	8.5 ± 0.8	9.9 ± 2.7	20.4 ± 2.8	31.0 ± 4.9
95% confidence interval	[7.9–9.1]	[7.9–11.9]	[18.3–22.5]	[27.3–34.7]
TEER (120 min, % of initial)	86 ± 6	77 ± 10	61 ± 11	49 ± 7
95% confidence interval	[83–88]	[73–82]	[56–66]	[46–53]
P-value (*t*-test relative to GR)[a]	–	0.45	5.9 × 10^{-9}	2.9 × 10^{-13}

[a] Average *P*-values after testing the TEER of all times (paired *t*-test) up to 120 min.

basolateral side (100 μl with refill) 10, 20, 30, 40, 60, 80, 100, 120 min after initiating the study. The samples were transferred to polyethylene tubes and added scintillation fluid (2 ml) Ultima Gold both purchased from Packard (Meriden, CT, USA) and analyzed by scintillation. TEER was measured after the basolateral sample withdrawal and replacements of volumes were performed.

2.6. Analytical methods

The analytical method was a modified HPLC method based on (Tietz et al., 1984). The HPLC system for GC analysis was from Merck and consisted of a Hitachi L-6000 pump and a Hitachi L-4000 UV detector connected to a Hitachi LaChrom L-7400 autosampler (plot attenuation = 4). The injector was a Rheodyne model 7125 (Berkeley, CA, USA) and the column was a 4 mm i.d. × 125 mm LiChrosorb RP-18, and the guard column was a LiChroCART 4-4, both from Merck. The mobile phase (adjusted to pH 2, by adding 75 ml l^{-1} 85% phosphoric acid) consisted of an aqueous solution of 50 mM sodium phosphate and 70% (v/v) methanol of analytical quality. The mobile phase was filtered through a Millipore 0.45 μm filter and degassed ultrasonically. The flow-rate was 1.0 ml min^{-1}. The excitation wavelength was 200 nm. Calibration standard curves were performed each day of analysis.

Samples from the mannitol transport study was counted for 10 min on a TriCarb-2100TR liquid scintillation counter form Packard (Meriden, CD, USA).

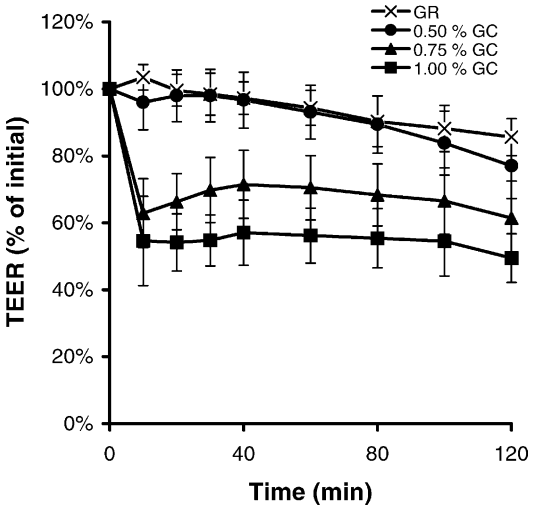

Fig. 3. Average transepithelial electrical resistance relative to initial in % across Caco-2 cell monolayers (n = 6, for each of three passages). The initial apical GC concentration was 0 (GR, control), 0.50, 0.75 and 1.00%, respectively.

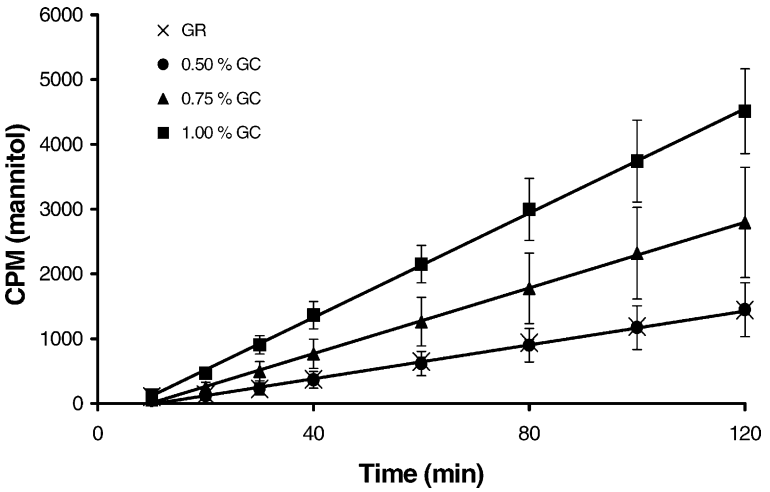

Fig. 2. Average transport profile for mannitol (CPM) across Caco-2 cell monolayers (n = 3, for each of three passages). The initial apical GC concentration was 0 (GR, control), 0.50, 0.75 and 1.00%, respectively.

2.7. Calculations

Two-sided ANOVA concerning the effect of passage and concentration was performed. Additional the mannitol data and the TEER (120 min) were evaluated by a two-sided Student's *t*-test. The correlation for dilution, when replacing the sampled volume followed the equation: $Q = V_s\left(\sum C_{n-1}\right) + C_n V_t$, where Q is the total amount of mannitol or GC, V_s denotes the sample volume, V_t the total volume and $C_{1,2,\dots,n}$ represents the concentration of sample $1, 2, \dots, n$. The permeability coefficient of GC and mannitol was calculated using the following equation:

$$P = \frac{dQ}{dt} \times \frac{1}{C_0 \times A}$$

where dQ/dt is the slope obtained from the permeability curve, C_0 denotes the initial apical concentration, and A is the area of the epithelial monolayer.

3. Results and discussion

The flux of GC was very low (all $<1\%\,h^{-1}$) with no significant difference between the three passages, however, concentration-dependent and linear with time (Fig. 1). The GC permeabilities (Papp) were 4.8, 12.9 and 25.8 ($\times 10^{-7}$ cm s^{-1}) for 0.50, 0.75 and 1.00% GC, respectively (Table 1). Mannitol transport changed accordingly with Papp of 8.5, 9.9, 20.4 and 31.0 ($\times 10^{-7}$ cm s^{-1}) for GR, 0.50, 0.75 and 1.00% GC, respectively (Table 1 and Fig. 2) with TEER after 120 min, relative to initial, at 86, 77, 61, and 49% (Table 1 and Fig. 3). The permeability of GC was even lower than for mannitol, and as a relatively linear relation exists between flux of GC and the paracellular marker mannitol for the various concentration levels of the enhancer (Fig. 4), it is indicated that the flux of GC may be dependent on the integrity of the tight junctions.

The results indicates that their is a linear concentration dependency, for the examined concentration levels, of GC on the opening of the tight junctions. Even though the molecular size of mannitol (MW 182) is somewhat lower than GC (MW 488) the relative effect on permeability is about the same. By extrapolating Papp (GC) as a function of GC concentration, the lowest effective level may be estimated to 0.41% (8.3 mM), which is in agreement with the very low and insignificant enhancer effect of the used 0.50% GC level on mannitol transport and TEER.

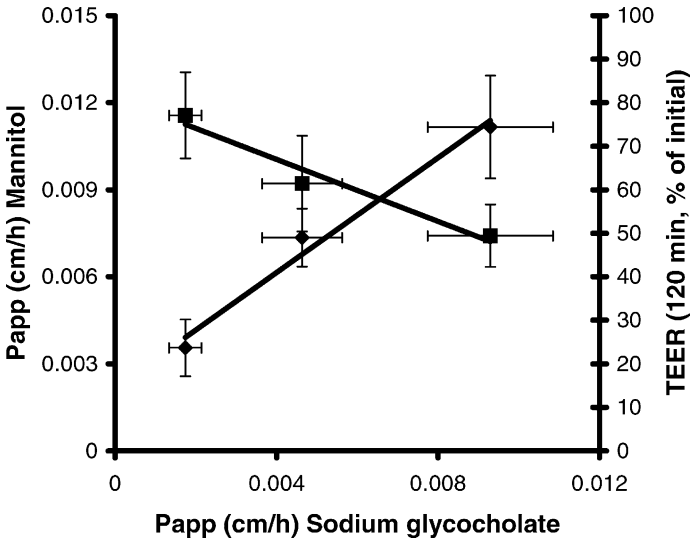

Fig. 4. Correlation of Papp (cm h^{-1}) for mannitol, Papp (cm h^{-1}) GC and TEER across Caco-2 cell monolayers ($n = 3$, for each of three passages). The initial apical GC concentration was 0 (GR, control), 0.50, 0.75 and 1.00%, respectively. Data for mannitol permeability and TEER relative to GC permeability is marked with the symbols (◆) and (■), respectively.

TEER after 120 min is lowered by the various concentrations of GC, according to the Papp of the enhancer and mannitol (Fig. 4), showing the close relation between the "tightness" of the junctions and the resistance across the Caco-2 cell monolayer. The TEER is decreased in less than 10 min to a certain level, without further reduction in a 120-min period, indicating that the enhancer effect is momentarily, rather than time-dependent. If the GC effect was due to a general toxic effect on the cell membranes, a time-dependent reduction of TEER would be expected. This is consistent with earlier results described by Jørgensen et al., where the effect of GC on Caco-2 cells was found to be reversible in concentration levels about 10 mM. Both mannitol transport and TEER was found to be useful tools and about equally sensitive, in testing the effect of GC on the cell monolayers.

Apparently, the experimental conditions have been well controlled with respect to apical concentration, basolateral stability and adsorption. Additionally, it was not found necessary to correct the GC permeability with respect to filter barrier properties, because the permeability across the filter, without cells, was more than two orders of magnitude higher than the permeability across the cells. The apical GC concentration was almost constant, however, with a small initial decrease (about 10%). This may be explained by accumulation of GC in the cell layer, which was reported for buccal tissue (Senel et al., 1998). The average total recovery of GC was $98 \pm 8\%$ and the basolateral stability was high, with average recoveries of $96 \pm 9\%$ for all concentrations.

In conclusion a low and concentration-dependent permeability was found for GC across the Caco-2 cells. Mannitol transport increased and TEER decreased accordingly. TEER decreased in less than 10 min to a certain level, without further reduction in a 120-min period, indicating that the enhancer effect is momentarily, rather than time-dependent. The apical GC concentration and enhancer effect may be considered well defined during the experiment, due to the observed low permeability of GC.

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