Quantification and Imaging of Mannitol Transport Through Caco-2 Cell Monolayers Using a Positron-Emitting Tracer

Lucia Lazorova,[1] Johan Gråsjö,[1] Per Artursson,[1,3] Mats Bergström,[2] Feng Wu,[2] Elisabeth Petterman-Bergström,[2] Mattias Ögren,[2] and Bengt Långström[2]

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INTRODUCTION

Monolayers of epithelial or endothelial cells are currently evaluated for their ability to predict *in vivo* permeability of the intestinal epithelium to drugs (1). However, only limited information is available with regard to the permeability profiles of commonly used permeability markers such as mannitol. Typically, the permeability of mannitol is monitored at late time points, i.e., after 20–60 min in drug transport studies and no information is available with regard to the lag time for the establishment of steady-state diffusion of mannitol across the monolayers. Such information would allow the determination of the minimal number of samples required to obtain reliable permeability coefficients. Moreover, no images of the distribution of common permeability markers in the entire monolayer during the diffusion process has been presented. Such images would give information of regional differences in marker permeability in individual monolayers. In this study, ^{11}C-labeled mannitol (a positron-emitting tracer (PET) of high specific radioactivity) was synthesised and used to determine the characteristics of mannitol in transport experiments using Snapwell cell culture inserts. The exclusive and relatively demanding PET-technique was used since PETs has a very high specific radioactivity. This allowed studies of the initial permeability of ^{11}C-mannitol in Caco-2 monolayers as well as imaging of the ^{11}C-mannitol distribution in the cell monolayers. Under controlled stirring conditions, the rate limiting barriers for the permeability of Caco-2 monolayers to ^{11}C-mannitol were determined. In particular, the lag time for the establishment of steady-state diffusion of ^{11}C-mannitol across Caco-2 cell monolayers was characterised. The use of ^{11}C-mannitol also allowed imaging of the distribution of the tracer during its transport over the entire cell monolayer for the first time.

[1] Department of Pharmaceutics, Uppsala University, Box 580, S-751 23 Uppsala, Sweden.

[2] Subfemtomole Biorecognition Project, Uppsala University PET Centre, University Hospital, S-751 85 Uppsala, Sweden.

[3] To whom correspondence should be addressed. (e-mail: per.artursson@galenik.uu.se)

MATERIALS AND METHODS

Synthesis of ^{11}C-mannitol

^{11}C-labeled carbon dioxide was produced by the ^{14}N(p,α)^{11}C nuclear reaction, using a Scanditronix MC-17 cyclotron at the Uppsala University PET Centre. The synthesis of D-[1-^{11}C]mannitol was undertaken using a semi-automated chemistry system (4). The radiochemical purity of D-[1-^{11}C]mannitol was determined by chromatographic separation on an anion exchange column and was in excess of 97%. The specific activities of different D-[1-^{11}C]mannitol batches at the end of synthesis were in the range of 2.8–3.5 GBq/μmol.

Cell Culture

The human intestinal epithelial cell line Caco-2 was obtained from the American Type Culture Collection (Rockville, MD). The cells (passage numbers 92–102) were cultivated as described elsewhere (2) on polycarbonate filters (Snapwell® or Transwell® cell culture inserts; 12 mm diameter, 0.4 μm pore size; Costar, Cambridge, MA). The cell monolayers were used in transport experiments 21–35 days after seeding. Monolayers sampled from each batch had a transepithelial electrical resistance of > 200 ohm.cm^2.

Transport of ^{11}C-mannitol Across Empty Filters

The Snapwell cell culture inserts were mounted in diffusion chambers (Precision Instrument Design, Tahoe City, CA), and 5 ml of Hank's Balanced Salt Solution containing 25 mM Hepes buffer, pH 7.4, (HBSS) pre-warmed to 37°C, was added to each side of the filter. The diffusion chambers were stirred continuously with an O_2/CO_2 (95/5%) gas mixture as described previously (5). After 30 min equilibration at 37°C, the desired volume of HBSS was removed from the donor chamber and replaced with an equal volume of buffer containing freshly synthesised ^{11}C-mannitol (final radioactivity 1 MBq/ml). At 20 s intervals, 1.0 ml samples were withdrawn from the acceptor chamber and immediately replaced with 1.0 ml of pre-heated HBSS. ^{11}C-mannitol radioactivity in the acceptor samples was analysed using four γ-scintillation counters of well type (custom made, Uppsala University PET Centre) (6).

Transport of ^{11}C-mannitol Through Caco-2 Monolayers

Monolayers grown on Snapwell cell culture inserts were washed with pre-warmed HBSS and transferred to the diffusion chambers, where they were allowed to equilibrate for 60 min at 37°C. The desired volume of HBSS was removed from the donor chamber and replaced with an equal volume of buffer containing freshly synthesised ^{11}C-mannitol (final radioactivity 10 MBq/ml). At 60 s intervals, 1.0 ml samples were withdrawn from the acceptor chamber and analyzed as described above. The experiments were repeated five times using 4-5 monolayers each time.

Imaging of ^{11}C-mannitol Transported Through Caco-2 Monolayers

Because of the short $t_{1/2}$ of ^{11}C (20.3 min), the transport and imaging experiments had to be performed on separate

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monolayers. Caco-2 cell monolayers grown on Transwell cell culture inserts were washed with pre-warmed HBSS and placed in 12-well cell culture clusters (Costar, Cambridge, MA). 0.4 ml of HBSS mixed with ^{11}C-mannitol (final radioactivity 5 MBq/filter) was added to the apical side and the monolayers were mildly agitated (100 rpm) at 37°C for 15 min. The filters were washed from both sides with 0.5 ml and 2.0 ml HBSS, respectively, by agitating at 100 rpm for 1 min and careful removal of all liquid from the edges with a tissue paper. Preliminary experiments showed that the tracer concentration and the washing procedure (e.g. the number of washings) only affected the intensity, not the distribution of the signal from ^{11}C-mannitol as detected by the phosphor imager. The monolayers were then placed into a custom-made cell culture cluster. To obtain optimal detection of the residual radioactivity, the bottom of the cell culture cluster was made of a thin cellulose acetate foil. 0.4 ml and 1.5 ml of pre-warmed HBSS was added to the apical and basolateral sides of the monolayers, respectively. The cluster containing the monolayers was placed on a storage phosphor (SP) plate (Molecular Dynamics, Sunnyvale, CA, USA) covered by a 10 μm sheet of plastic foil. After exposure at 21°C for different time intervals, the plates were scanned in a Phosphor Imager (Model 400S, Molecular Dynamics) using a 10 mW HeNe laser beam (6). The experiments were repeated five times using eight monolayers each time.

Theory

Determination of Permeability Coefficients and Lag Time

The permeability coefficient was calculated from the linear equation

$$P_{app} = \frac{dC_R}{dt} \cdot \frac{1}{C_D} \cdot \frac{V_R}{A} \approx \frac{dC_R/C_D}{dt} \cdot \frac{V_R}{A} \Rightarrow \frac{C_R}{C_D} \approx P_{app} \cdot \frac{A}{V_R} \cdot t \quad (1)$$

where P_{app} is the apparent permeability, C_R is the concentration in the receiver compartment, C_D is the concentration in the donor compartment, V_R is the volume of the receiver compartment, A is the area of the filter and t is the time from addition of mannitol. The advantage of using equation 1 is that it takes the concentration changes in the donor compartment into consideration. Linear curve fitting of C_R / C_D vs t gives the slope k, the intercept with the time axis gives the lag time (t_{lag}), and P_{app} is obtained from $P_{app} = k \cdot V_R/A$.

The observed permeability of the system to mannitol (P_{app}) is the resultant permeability of serially arranged barriers: the aqueous boundary layer, the empty filter and the epithelium, as expressed by

$$\frac{1}{P_{app}} = \frac{1}{P_{ABL}} + \frac{1}{P_f} + \frac{1}{P_c} \quad (2)$$

where P_{ABL} is the permeability of the aqueous boundary layer, P_f is the filter permeability; and P_c is the cell monolayer permeability (7).

Karlsson *et al.* (7) have suggested a method of calculating P_f. Using this method assume that the aqueous diffusion coefficient (D_{aq}) for mannitol at 37°C is 9.1 × 10^{-6} cm^2/s, P_f = 1.2 × 10^{-3} cm/s.

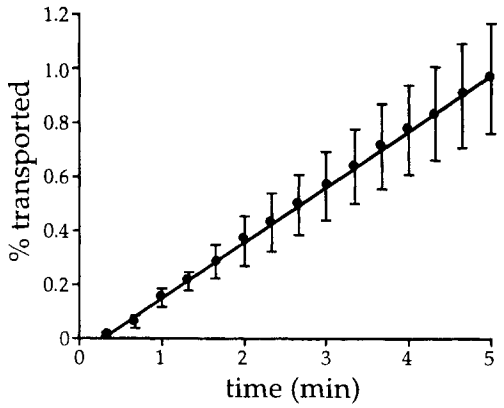

Fig. 1. Transport of ^{11}C-mannitol across empty Snapwell filters. Values are expressed as % of the initial radioactivity in the receiver compartment (mean ± s.d., n = 5). The t_{lag} was determined from the intercept of the graph with the time axis.

Theoretical Explanation of t_{lag} Originating from Diffusion Through the Barrier

For a barrier where a substance in the donor compartment is kept at a constant concentration C_D and the receiver compartment is kept under sink conditions, the amount of transported substance (M) to the receiver compartment is given by the equation

$$M(t) = A \cdot \left(\frac{DC_0 t}{h} - \frac{2hC_0}{\pi^2} \sum_{n=1}^{\infty} \frac{(-1)^n}{n} \cdot e^{-n^2\pi^2 Dt/h^2} - \frac{hC_0}{6} \right) \quad (3)$$

where D is the diffusion coefficient, C_0 is the concentration in the barrier at the donor side, h is the thickness of the barrier, and t is the time (8).

If t is large, the middle term in equation 3 will be vanishingly small. Thus, when t is large, equation 3 becomes:

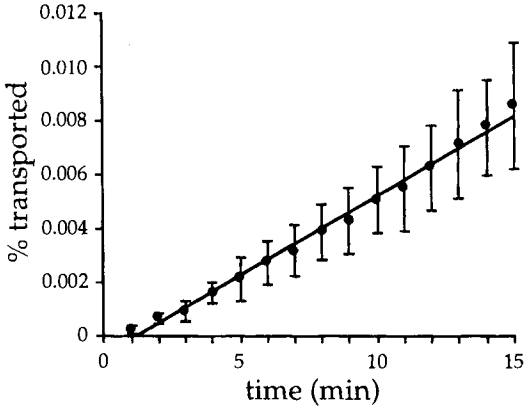

Fig. 2. Transport of ^{11}C-mannitol across Caco-2 cell monolayers. Values are expressed as % of the initial radioactivity in the receiver compartment (mean ± s.d., n = 4). The t_{lag} was determined from the intercept of the graph with the time axis.

$$M(t) = \frac{A \cdot C_0}{h}\left(D \cdot t - \frac{h^2}{6}\right) \quad (4)$$

Equation 4 is a straight line with the slope k = A C_0 D/h, which intersects the time axis (t_{lag}) when ($D \cdot t - h^2/6$) = 0, i.e.

$$t_{lag} = h^2/6 \cdot D \quad (5)$$

RESULTS AND DISCUSSION

The results of this study show that PETs of high specific radioactivity can be used to obtain drug permeability profiles of high resolution as well as to image the distribution of the tracer in the cell monolayer. The potential of tomographic images obtained with PET will allow an extension of results obtained in Caco-2 cell studies to permeability studies in experimental animals and in man. This will enable quantitative comparisons of *in vitro* and *in vivo* permeabilities for a variety of PETs, including drugs, peptides and oligonucleotides.

The high specific radioactivity of ^{11}C-mannitol allowed the determination of transported radioactivity across empty filters with high precision, Figure 1. P_{app} was 1.6 ± 0.4 × 10^{-4}cm/s (n = 5), which, although high, should be compared with the theoretical value of 1.2 × 10^{-3} cm/s, i.e. 7.4-fold higher (7). It was calculated that this difference was a result of the barrier caused by the aqueous boundary layer (5). Linear regression of the transported amount of ^{11}C-mannitol vs time gave an intercept (t_{lag}) with the time axis at 19 ± 4 s, Figure 1. According to equation 5, diffusion through the empty filter would give a t_{lag} of 0.018 s only (8). The main contribution to t_{lag} was related to the time taken to obtain complete mixing of ^{11}C-mannitol in the donor and receiver chambers. At least 7 seconds were required to obtain complete mixing in the donor chamber in studies of mixing which used phenol red as a marker, even under the vigorous stirring conditions used in the present study (data not shown). Thus, it can be expected that the total time for complete mixing in both chambers will be in the vicinity of the observed t_{lag}.

The P_{app} of Snapwell filters with Caco-2 monolayers to ^{11}C-mannitol was 4.4 ± 1.1 × 10^{-7} cm/s (n = 4). Since both P_f and P_{ABL} are much larger than P_{app} under these conditions, the influence of the permeabilities of these two barriers on P_{app} can be disregarded and $P_{app} = P_c$, equation 2. This P_c value is in the range of previously reported P_c values for ^{11}C-mannitol (9, 10). The P_{app} of phospholipid bilayers for ^{11}C-mannitol has recently been determined to be in the range of 5 × 10^{-11} cm/s (11) or about 10,000-fold lower than the corresponding P_{app} for Caco-2 monolayers. It is therefore reasonable to assume that ^{11}C-mannitol permeated the cell monolayers exclusively through the aqueous pores of the paracellular pathway.

Linear regression of the amount of ^{11}C-mannitol transported vs time gave an intercept (t_{lag}) with the time axis at 84 ± 17 s, Figure 2. According to the results using empty filters, about 19 s of the t_{lag} was accounted for as time for mixing of ^{11}C-mannitol in the donor and receiver chambers. The remainder of t_{lag} was therefore the time for establishment of steady-state diffusion in the paracellular pathway. The short t_{lag} for mannitol observed during thorough stirring in the present study, indicates that the t_{lag} can be disregarded in normal experimental settings and that the permeability of Caco-2 cell monolayers to mannitol can be determined using a single point measurement. Thus, using linear regression and the intercept with the time axis in Figure 2, the P_{app} of the Caco-2 cells to mannitol is 4.4 ± 1.1 × 10^{-7} cm/s. This value is not significantly different from the P_{app} value of 4.2 ± 1.1 × 10^{-7} cm/s, which is obtained if the t_{lag} is replaced by origo in the calculations. This information can be used to minimize the sampling of this integrity marker and should be especially useful when the Caco-2 cell culture technology is adopted to the screening mode.

Images of ^{11}C-mannitol distribution within the in Caco-2 monolayers showed a surprisingly homogeneous radioactivity across the entire surface of the monolayers, Figure 3A. Importantly, the radioactivity was not increased at the border between the cell monolayers and the plastic of the cell culture inserts, suggesting an intact cell-plastic barrier to mannitol comparable to the tight junction barrier between the cells. One of the cell monolayers was significantly more permeable to mannitol than the other monolayers, Figure 3B. Images of this monolayer at a lower detection level showed an area of increased radioactivity, Figure 3C. We tentatively conclude that this monolayer was injured during the cultivation period preceding the use of the monolayers in the transport studies, e.g. through direct contact with the suction pipette used for replacement of the cell culture medium.

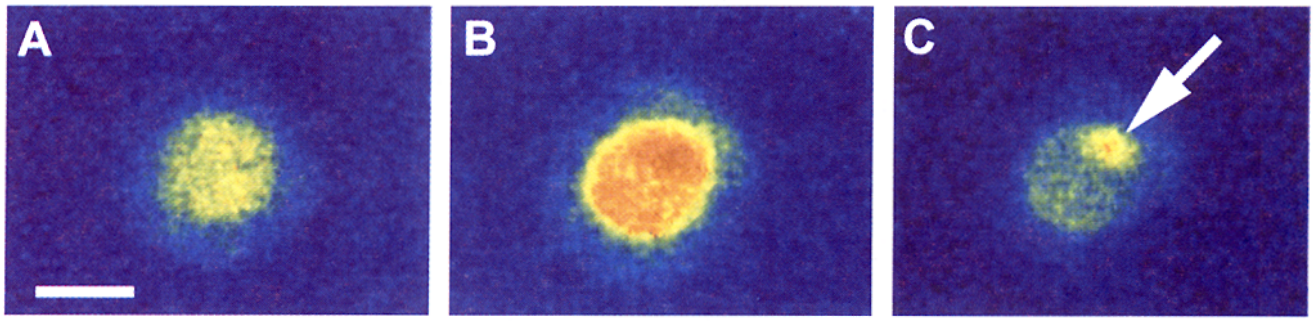

Fig. 3. Images of ^{11}C-mannitol distribution within the Caco-2 monolayers. Entire monolayers are shown. A. Image of an intact monolayer. An even distribution of the transported radioactivity is observed; B. image of a monolayer with enhanced permeability; C. the same monolayer as in B but depicted after a shorter exposure time. Note a hot red spot with a local increase in transported radioactivity (arrow-head). The bar indicates 1 cm.

Although the present study indicates that positron-emitting tracers are powerful tools in *in vitro* studies of the permeability of cell monolayers to drugs, the application of these tracers also has several limitations. The rapid half-life of ^{11}C requires a dedicated laboratory for rapid synthesis and purification of ^{11}C-markers and means that studies must be completed in very short time periods (12–13). In this study, the physical half-life of ^{11}C was 20.4 min. It may be possible in some situations to use other positron-emitting radionuclides such as ^{18}F, ^{76}Br, and ^{124}I (half-lives 110 min, 16.3 hours, and 4.3 days, respectively) depending on the drug under investigation. Nevertheless, this first study on the application of a positron-emitting tracer in transport experiments in cell culture suggests that such tracers can generate new and valuable information on drug permeability *in vitro*.

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